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Role of Retinoids in the Regulation of Hepatic Glucose and Lipid Metabolism

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To the Graduate Council:

I am submitting herewith a dissertation written by Rui Li entitled "Role of Retinoids in the Regulation of Hepatic Glucose and Lipid Metabolism." I have examined the final electronic copy of this dissertation for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Doctor of Philosophy, with a major in Nutritional Sciences.

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Role of Retinoids in the Regulation of Hepatic Glucose and Lipid Metabolism

A Dissertation Presented for the

Doctor of Philosophy

Degree

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Rui Li

May 2013

To My Parents

Qibin Li and Huiqin Ma

For Their Constant Love and Support

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ABSTRACT

The liver plays an important role in controlling glucose and lipid homeostasis. Metabolic abnormalities such as obesity and type 2 diabetes are often associated with profound changes in the expression of genes involved in hepatic glucose and lipid metabolism. Dietary nutrients provide us with macronutrients for energy and micronutrients for maintenance of general health. However, the effects of individual micronutrients on the development of metabolic diseases are unknown. Sterol regulatory element binding protein-1c (SREBP-1c) is the master regulator of fatty acid synthesis, and glucokinase (GK) is the key enzyme in glucose metabolism. Based on the preliminary results from our laboratory and others, we hypothesized that the metabolism of vitamin A (VA) regulate lipid and glucose metabolism by controlling the expression of *Srebp-1c* and *Gck* in hepatocytes. We show that retinoids synergize with insulin to induce the expression of *Srebp-1c* and *Gck* in primary rat hepatocytes. Retinoid-induced expression of *Srebp-1c*, due to the activation of RXR, results in increased expression of SREBP-1c target gene, *Fas*. Results obtained from the reporter gene assays demonstrate that the retinoic acid responsive elements (RAREs) in the *Srebp-1c* promoter overlap previously identified liver X receptor elements that mediate insulin action. For hepatic *Gck*, we first characterized its promoter and identified a RARE using serial deletion reporter gene assays and linker-scan analyses. This RARE overlaps a putative binding site for HNF4α [hepatic nuclear factor 4 alpha]. We then performed chromatin immunoprecipitation and electrophoretic mobility shift assays to study the binding of this RARE to nuclear receptors (NRs) potentially mediating retinoid actions: RARα [retinoic acid receptor alpha], RXRα [retinoid X receptor alpha], HNF4α, and COUP-TFII. We show that these NRs are capable of binding to the RARE and their binding activities are modulated by retinoic acid. In addition, the effects of the recombinant adenovirus-mediated over-expression of these NRs on the expression of retinoids- and insulin-responsive genes were analyzed in primary

hepatocytes. We observed profound modulation of the gene expression by these NRs. We conclude that VA can control hepatic glucose and lipid metabolism via regulation of the expression of genes involved.

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CHAPTER I
INTRODUCTION

The significant increase in the prevalence of obesity and diabetes is an important health issue worldwide. According to the World Health Organization (WHO) approximately 1.6 billion adults worldwide were overweight (body mass index, BMI, between 25 and 30 kg/ m^2) in 2005 and at least 400 million adults were obese (BMI of 30 kg/ m^2 or higher). The numbers will continue to rise and WHO's projections estimate that by 2015, approximately 2.3 billion adults will be overweight and more than 700 million will be obese [1]. In the United States, the current prevalence of obesity among adults is about 33%, compared to estimates four decades earlier showing a prevalence of about 13% [2]. Unfortunately, obesity is not exclusively a problem in adults. More than one third of children and adolescents in the United States were overweight or obese in 2008 [3]. In addition, the obesity epidemic is imposing a significant economic burden on healthcare system globally. It is estimated that obesity accounts for between 0.7% and 2.8% of a country's total healthcare expenditures. Moreover, obese individuals are found to have medical costs that are approximately 30% greater than their normal weight peers [4].

Obesity is associated with increased risk of many chronic diseases, including type 2 diabetes (T2D), cardiovascular disease, and many forms of cancer [5]. T2D affects approximately 6% of the world's adult population and this number will continue to soar with the increase of obese population. In North America, about 90% of people with T2D are obese, overweight, or have a medical history of being so [6]. Co-existence of obesity and T2D, which poses a challenge to the treatment of both conditions, is attributed to both genetics (i.e. genes that affect feeding behavior and metabolism) and environment (i.e. excess calories, sedentary lifestyles, etc.). Although the exact mechanism linking increased risk of T2D to obesity is not completely understood, insulin resistance, a common feature of obesity and T2D, is considered to play an important role. Genetic and environmental factors confer susceptibility to excess adiposity, which in turn promote insulin resistance. Insulin resistance is initially compensated for by increased insulin

secretion from pancreatic β cells. Hyperglycemia develops when insulin secretion is unable to overcome the insulin resistance and continued deterioration of pancreatic β cell function causes further impairment of glucose homoeostasis into T2D [6].

The liver plays a critical role in mediating glucose and lipid homeostasis in response to hormonal and nutritional stimuli. In the liver and hepatocytes, insulin regulates the expression of a variety of genes responsible for glycolysis, glycogenesis and lipogenesis, and inhibits gluconeogenesis. This insulin-regulated hepatic gene expression, at least in part, is responsible for glucose and lipid homeostasis [7]. For example, insulin increases the expression of glucokinase gene (*Gck*), the enzyme responsible for the first step of hepatic glucose utilization. It suppresses the expression of the cytosolic form of phosphoenolpyruvate carboxykinase (*Pck1*) and glucose 6-phosphatase catalytic subunit (*G6pc*), the first and last steps of gluconeogenesis, respectively. For hepatic lipid metabolism, insulin increases the expression levels of sterol regulatory element binding protein 1c gene (*Srebp-1c*), a master regulator of fatty acid synthesis [8]. In hepatic insulin resistance, gluconeogenesis is not adequately suppressed by insulin, whereas the lipogenic actions of insulin is not compromised, thus creating a vicious cycle that aggravated insulin resistance and ultimately contributes to the onset of overt diabetes [9].

Dietary nutrients provide us with macronutrients for energy and micronutrients for maintenance of general health [7]. The link between obesity development and over-nutrition seems to be obvious. However, the exact roles of each micronutrient in the development of metabolic diseases have not been clearly defined. Vitamin A (VA, retinol), a micronutrient, plays crucial roles in many physiological processes, including embryonic growth, development, tissue differentiation, and nutrient metabolism [10]. Previously, our lab demonstrated a synergistic effect between retinoids and insulin to induce *Gck* and *Srebp-1c* expression in primary rat hepatocytes [11, 12]. This dissertation work was to investigate the underlying mechanism. Its results

may shed some lights on our understanding of the development of hepatic insulin resistance.

CHAPTER II
LITERATURE REVIEW

2.1 Obesity

2.1.1 Obesity Overview

The survival of all organisms depends on continuous availability of energy despite highly variable energy supplies in the environment [13]. Mammals including human have acquired a robust physiological system to promote efficient storage of unused energy in the form of triglycerides (TGs) in specialized adipose cells, from which stored energy could be rapidly released for use by other cells upon starvation [13]. It has been suggested that body weight (BW) and energy balance (EB), the long-term balance between energy intake (EI) and energy expenditure (EE) [14], are delicately regulated [15]. Obesity results from a chronic surplus of EI compared to energy expenditure (EE), which leads to storage of excessive amounts of TGs in adipose tissue [16]. EE is consisting of the energy consumed in the processes of physical work, basal metabolism, and adaptive thermogenesis [14]. In a consequence of social trends toward high EI and reduced EE [17], obesity has reached epidemic proportions globally (see Chapter I). Obesity is associated with increased risk of T2D, cardiovascular disease, and many types of cancer, fatty liver disease, hormonal disturbances, hypertension, and increased mortality [18]. In addition, obesity is associated with respiratory difficulties, chronic musculoskeletal problems, lumbago, skin problem, and infertility. It is noteworthy that most of the evidence proposing obesity-associated health problems has been obtained from epidemiological analyses of human subjects and the underlying mechanisms have yet to be revealed [5]. Despite current intensive efforts to reduce obesity by diet, exercise, education, surgery, and drug therapies, an effective, long-term solution to this epidemic is yet to be established [18].

2.1.2 EB and BW

EB is reached when the EI is equal to EE. Although the role of genes in body fat regulation is well established, it is unlikely that the increasing prevalence of obesity is due to a recent genetic change of the Western world

[14]. In fact, the obesity epidemic is considered on a large scale to result from changes of lifestyle, including increased fat intake and reduced physical activity. It has been suggested that a physiological system in human body functions to maintain homeostasis of energy stores in response to variable access to nutrition and demands for EE [19]. Any perturbation of the component of the EB system can lead to abnormal weight.

The regulation of EI has been considered a coordinate response of the energy storage inside the body and environmental stimuli. The hypothalamus is a major site for interpretation and integration of central and peripheral signals that regulate energy homeostasis. Lesions in the ventromedial hypothalamus cause hyperphagia and obesity, lesions in the lateral hypothalamus cause aphagia and leanness and even death by starvation [20]. Within the hypothalamus, neurons residing in the ARC-PVN-PF/LH axis communicate among each other and are subjected to the influence of several peripheral factors, such as leptin and insulin [21]. Fig. 2.1 summarizes the importance of hypothalamus in regulating energy homeostasis.

2.1.3 Genetics of Obesity

Although a large percentage of human obesity is attributed to adverse lifestyle, it is well known that genetic variation among individuals influences responses to environmental factors such as food intake and physical activity. In the 1960s, Neel proposed the ‘thrifty gene’ hypothesis, whereby genes that predispose to obesity would have had a selective advantage in populations that frequently experienced starvation. People who possess these genes in the current obesogenic environment might be overweight or obese [22]. It is estimated that heritability for BMI ranges between 50-70%, while heritability for total body fat is as high as 80% [23]. These numbers, along with twin studies that provide the most powerful test of heritability, demonstrate that obesity is partially genetically regulated.

Obesity is commonly classified into subgroups depending on suspected etiology: monogenic obesity, syndromic obesity, and polygenic or common

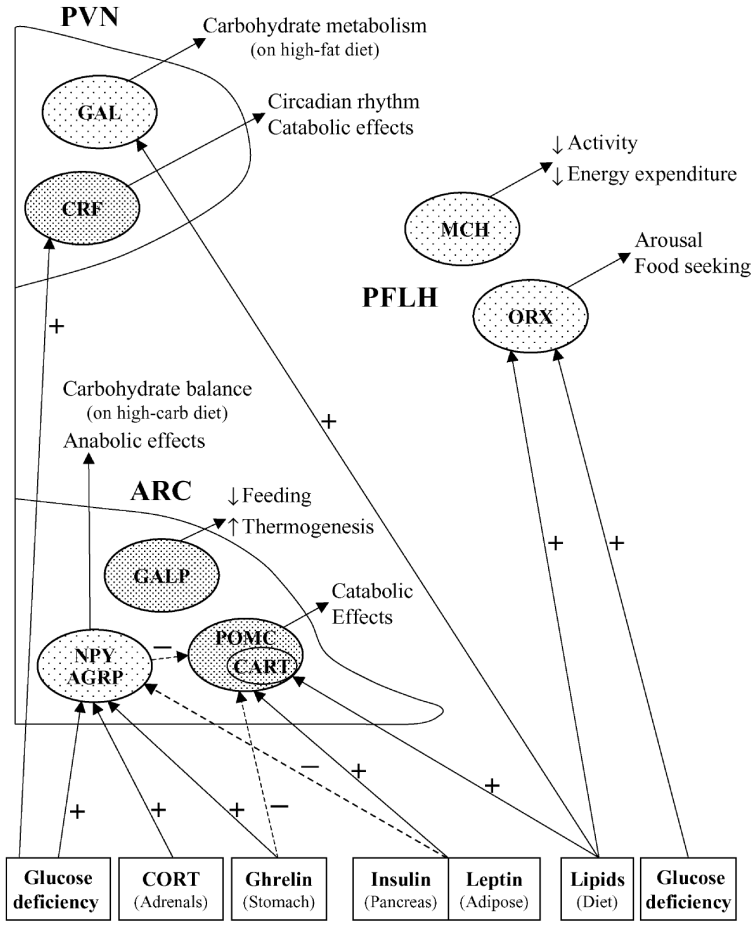

Figure 2.1 Hypothesized model of nine peptide systems in the hypothalamus involved in energy homeostasis

Signals shown at the bottom, which control the expression and production of these peptides, are indicated with arrows as a stimulatory effect (+) or inhibitory effect (–). The behavioral and physiological actions of these peptides are also shown. Hypothalamic areas represented are the pariventricular nucleus (PVN), perifornical area + lateral hypothalamus (PFLH), and arcuate nucleus (ARC). The nine peptides are: AgRP, agouti-related protein; CART, cocaine- and amphetamine-regulated transcript; CRF, corticotropin-releasing factor; GAL, galanin; GALP, galanin-like peptide; MCH, melanin-concentrating hormone; NPY, neuropeptide Y; ORX, orexins; and POMC, pro-opiomelanocortin. Adopted from Leibowitz *et al.*, 2004 [24].

obesity [18].

2.1.3.1 Monogenic Obesity

Monogenic obesity is characterized by extremely severe phenotypes in the absence of developmental delays [18]. The first human single gene defect, which leads to leptin deficiency and causes monogenic obesity, was discovered in 1997 [25, 26]. Subsequently, variations in other genes in the Leptin-Melanocortin pathway were identified as causative of obesity. Additionally, mutations in three genes (*SIM1*, *BDNF*, and *NTRK2*) involved in neural development have been shown to cause rare monogenic obesity [27]. Currently, there are at least 20 single gene disorders that result in an autosomal form of human obesity [16]. All these genes control the central sensing of EB and their mutations lead to obesity due to increased appetite and reduced satiety.

2.1.3.2 Syndromic Obesity

There are about 30 different Mendelian disorders in which patients are clinically obese and additionally distinguished by mental retardation, dysmorphic features, and organ-specific developmental abnormalities. These syndromes, syndromic obesity, arise from discrete genetic defects or chromosomal abnormalities, and can be autosomal or X-linked. The most common disorders known are Prader-Willi syndrome (PWS), Bardet-Biedl syndrome (BBS), and Alström syndrome [28]. PWS, caused by a chromosomal abnormality of an imprinted region on chromosome 15q11-q12, is characterized by early-onset obesity resulting from hyperphagia caused by CNS dysfunction [29]. BBS is characterized by early-onset obesity and rod-cone dystrophy, morphological finger abnormalities, learning difficulties, and renal disease, among other clinical traits. BBS has been associated with at least 11 different chromosomal locations, including 11q13, 16q21, 3p13, 15q22.3, 2q31, 20p12, 4q27, 14q32.11, 7p14, 12q21.2, and 9q33.1 [28].

2.1.3.3 Polygenic or Common Obesity

Polygenic obesity arises when an individual's genetic makeup is susceptible to an environment that promotes EI over EE. In contrast to monogenic obesity, many genes and chromosomal regions contribute to defining the common obese phenotype. These genes have been implicated in a wide variety of biological functions, such as the regulation of food intake, EE, lipid and glucose metabolism, and adipose tissue development [28]. Three main strategies have been adopted in genome-wide association studies of genetic variants influencing BMI and obesity: a) genome-wide association studies of population-based samples to examine the full range of BMI values; b) case-control analysis of persons selected from the extremes of the BMI distribution; and c) genome-wide analyses of pattern of fat distribution, prompted by the particularly deleterious health effects of visceral fat accumulation [17]. These strategies have led to the identification of a number of common genetic variations (see review by McCarthy [17]).

2.2 T2D

2.2.1 Diabetes Overview

Diabetes is a condition defined by a state of chronic elevation of plasma glucose levels. There are two main types of diabetes. Type 1 diabetes (T1D), or insulin-dependent diabetes mellitus (IDDM), is mainly diagnosed during childhood or adolescence and results from an autoimmune destruction of insulin-producing pancreatic β cells by CD4+ and CD8+ cells and macrophages infiltrating the islet. T2D, or non-insulin dependent diabetes mellitus (NIDDM), constitutes over 90% of all diabetes cases. In humans, it has mostly been diagnosed after age of forty, although recently much younger cases are being reported. Unlike T1D in which insulin production nearly vanishes, insulin in T2D is usually produced, but cannot be properly utilized due to insulin-resistance in target cells [1].

T1D accounts for about 10% of all cases of diabetes. However, the prevalence of T1D has been globally rising during the past decades by as

much as 5.3% annually in the United States. It is estimated that T1D is responsible for $14.4 billion in medical costs and lost income each year in the United States [30]. Although there are rare monogenic forms of T1D, the common form is thought to be determined by the actions, and possible interactions, of multiple genetic and environmental factors [31]. Like obesity, genetic studies have identified a number of genes as important genetic susceptibility factors. For instance, the major susceptibility locus has been mapped to the HLA class II genes at 6p21 and accounts for up to 30-50% of genetic T1D risk [32]. However, identification of environmental factors has been difficult, mainly due to the wide gap between initiation and detection of ongoing diabetogenic events [31]. Viral infection has been considered as a popular candidate. It is proposed that decreased exposure to widespread infection of developing immune system of the infants may contribute to the increases in T1D incidence [33].

2.2.2 Insulin Resistance, β-cell Dysfunction, and T2D

Insulin resistance and β-cell dysfunction are two common processes underlying the development of type 2 diabetes. It has been clear that most insulin resistant individuals do not develop hyperglycemia. Under normal conditions, the pancreatic islet β-cells increase insulin release sufficiently to overcome the reduced efficiency of insulin action, thereby maintaining normal glucose tolerance. For insulin resistance to be associated with T2D, β-cell must be unable to compensate fully for decreased insulin sensitivity [34].

2.2.2.1 Insulin Signaling

Tight control of blood glucose in humans is dependent on the balance between glucose absorption from the intestine, production by the liver, and uptake by peripheral tissues. Insulin plays a critical role in regulating blood glucose concentration by increasing glucose uptake in muscle and fat, and inhibiting hepatic glucose production. In addition, insulin stimulates cell growth and differentiation, and promotes the storage of substrates in fat, liver, and

muscle by stimulating lipogenesis, glycogenesis, and protein synthesis, and inhibiting lipolysis, glycogenolysis, and protein breakdown. Insulin resistance or deficiency results in profound dysregulation of these processes, and produces elevations in fasting and postprandial glucose and lipid levels [35].

Insulin signaling is triggered by the binding of insulin to its receptor in the plasma membrane of the cell (Fig. 2.2). The insulin receptor is a heterotetrameric complex, consisting of 2 extracellular α subunits that bind insulin and 2 transmembrane β subunits with tyrosine kinase activity. Insulin binding to the α subunit induces the transphosphorylation of one β subunit by another on specific tyrosine residues in an activation loop, resulting in the increased catalytic activity of the kinase. The receptor also undergoes autophosphorylation at other tyrosine residues in the juxtamembrane regions and intracellular tail. The activated insulin receptor then phosphorylates tyrosine residues on intracellular substrates that include the insulin receptor substrate family (IRS1-4), IRS5/DOK4, IRS/DOK5, Gab-1, Cb1, APS and Shc isoforms, and signal regulatory protein family members. Once phosphorylated, these substrates act as docking molecules for proteins that contain Src homology region 2 (SH2) domains, which in turn become activated or associated with other downstream signaling molecules, setting off a complicated cascade of events [36, 37]. Recently, structural analysis of the insulin-insulin receptor complex revealed that the carboxy-terminal α-chain segment is critical to the hormone-receptor recognition [38].

Activation of protein kinase B (Akt/PKB) is a critical step in insulin signal transduction cascade. The effects of insulin on glucose metabolism are mediated by phosphatidylinositol (PI) 3-kinase. Upon tyrosine phosphorylation, IRS proteins interact with the regulatory subunit of PI3-kinase and activate the enzyme. Activated PI3-kinase in turn activates Akt/PKB, which phosphorylates and deactivates glycogen synthase kinase 3, leading to the activation of glycogen synthase and stimulation of glycogen synthesis. Another target of Akt/PKB is forkhead box protein O1 (FOXO-1). Phosphorylation of FOXO-1 by Akt/PKB leads to its exclusion from the

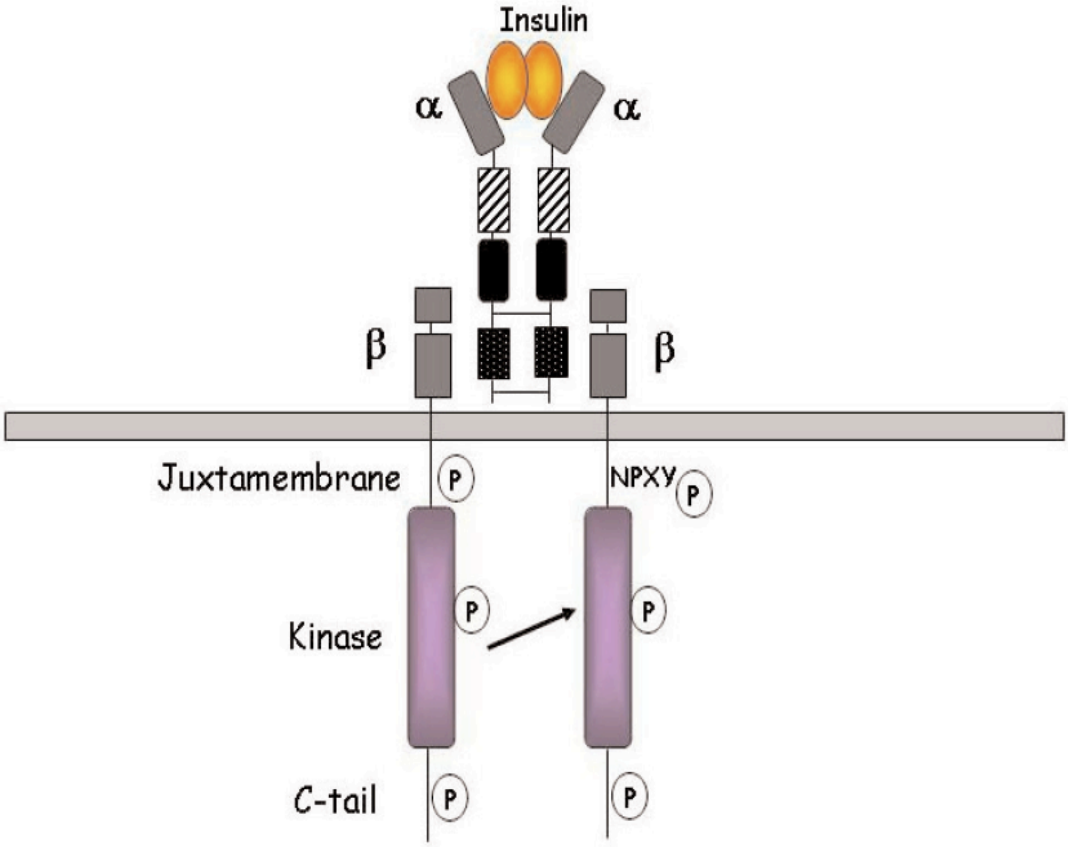

Figure 2.2 Structure of insulin receptor

See text for details. Adopted from Chang *et al.*, 2004 **[36]**.

nucleus, preventing it from activating transcription of *Pck1* and *G6pc* (see also Chapter I). In addition, Akt/PKB is essential to glucose uptake and glucose transporter 4 (Glut4) translocation as insulin-stimulated Glut4 translocation was inhibited by expression of a dominant-interfering Akt/PKB mutant [37]. However, it was recently shown that Akt/PKB was dispensable for insulin- and nutrient-mediated hepatic metabolic regulation *in vivo* [39].

It is considered that many of insulin's effects on lipogenesis are mediated by SREBP-1c (see Section 2.6).

2.2.2.2 Insulin Resistance

Insulin resistance is a common characteristic of obesity, T2D, and components of cardiometabolic syndrome, including hypertension and dyslipidemia [40]. It was proposed that the cluster of insulin resistance, impaired glucose tolerance, abnormalities of plasma lipids, and hypertension were part of a single syndrome, Syndrome X (also called insulin resistance syndrome, or metabolic syndrome) [41]. Insulin resistance is a decreased ability of some of the insulin-regulated processes and tissues (liver, muscle, and adipose tissue) to respond to insulin.

Liver plays a central role in glucose and lipid metabolism. In the liver, insulin regulates fasting glucose concentrations by inhibiting hepatic glucose production and stimulating glycogen synthesis. Hepatic glucose production involves two different mechanisms: glycogenolysis and gluconeogenesis. Glycogenolysis produces glucose during a relatively short-term fast of up to several hours, and is suppressed by insulin within 1-2 h after food intake under normal physiological conditions. During long periods of fasting (> 12-14 h), liver glycogen stores become depleted and there is an increase in the percentage contribution made by gluconeogenesis to the total glucose supply. The expression of two key enzymes for gluconeogenesis, PEPCK and G6Pase, is suppressed by insulin (see also Chapter I). However, this action is compromised in insulin resistance status.

Insulin also promotes the synthesis and inhibits the oxidation of fatty acids in the liver. When delivered to the liver in large quantities, glucose is first converted to glycogen and stored. Once glycogen stores are replenished, glucose enters the glycolysis pathway and thereby provides carbons for de novo lipogenesis. Lipids are then stored as triglycerides or exported from the liver as very-low-density lipoprotein (VLDL). The effects of insulin on hepatic lipogenesis are mediated by SRBEP-1c. Insulin induces the expression of *Srebp-1c* and key lipogenic genes, including fatty acid synthase (*Fas*), stearoyl-coenzyme A desaturase 1 (*Scd1*), and acetyl-CoA carboxylase (*Acc*) [42]. However, even in the presence of marked insulin resistance, hepatic transcription of *Srebp-1c* and lipogenesis are still stimulated by insulin, resulting in unaffected rates of de novo fatty acid synthesis [43]. The failure to suppress gluconeogenesis coupled with decreased peripheral glucose uptake accentuates the hyperglycemia and increases insulin secretion, further stimulating lipogenesis, resulting in a vicious cycle.

Several molecular mechanisms of insulin resistance have been proposed [37]. For examples, insulin resistance can be caused by impairments of insulin signaling cascade, which can be acquired through increased degradation, decreased expression, and/or decreased activity of major components of insulin signaling cascade, or through the interaction of inhibitory proteins with major components of insulin signaling cascade, such as the suppressors of cytokine signaling (SOCS) proteins that are induced by inflammatory cytokines. In addition, insulin resistance can be due to increased activity or amount of the enzymes reversing insulin action, such as phosphatase and tensin homolog and SH2 domain-containing inositol phosphatase [37].

2.2.2.3 β-cell Dysfunction

Although obesity often leads to insulin resistance, only a subset of obese, insulin resistant individuals progress to T2D. In both animal models and human, the triggering factor is β-cell failure, which involves a decrease in β-

cell mass and deterioration of key β-cell functions such as glucose-stimulated insulin secretion (GSIS).

At the very beginning of the progression towards T2D, increased plasma glucose due to insulin resistance is balanced by increased insulin secretion, which is mainly resulted from increased β-cell number, despite that β-cell hypertrophy may also contribute. As β-cells are no longer able to be compensating and normal glucose levels can no longer be maintained, loss of acute GSIS occurs (glucose toxicity). The loss of acute GSIS is accompanied by marked changes in β-cell phenotype demonstrated by changes in gene and protein expression (i.e. the highly expressed genes in β-cells are down-regulated while those that are normally suppressed are up-regulated in their expression). The glucose toxicity (glucotoxic effect), together with elevated plasma non-esterified fatty acid concentrations (lipotoxic effect), act synergistically to produce further deleterious effects on insulin, leading to continued β-cell loss and less efficient insulin secretion in T2D [34]. It has been shown that people with T2D have ~50% less of β-cell mass compared with control subjects. The reduction of the capacity of β-cell secretion is even more considerable [44].

A number of different hypotheses have been advanced as explanations for the development of β-cell dysfunction in T2D. One of the hypotheses is shown in Fig. 2.3.

2.3 GK in the Regulation of Glucose Homeostasis

2.3.1 Introduction of Glucose Metabolism

Glucose is of central metabolic importance in virtually all organisms, from microbes to humans. Glycolytic metabolism of glucose is a major pathway for the generation of energy (ATP), and glycolytic intermediates also serve as precursors for biosynthesis of other cellular constituents. Metabolism of glucose through the pentose phosphate pathway generates NADPH and precursors required for a variety of anabolic pathways. Alternatively, glucose may be converted to its polymeric forms (glycogen, starch), which are the

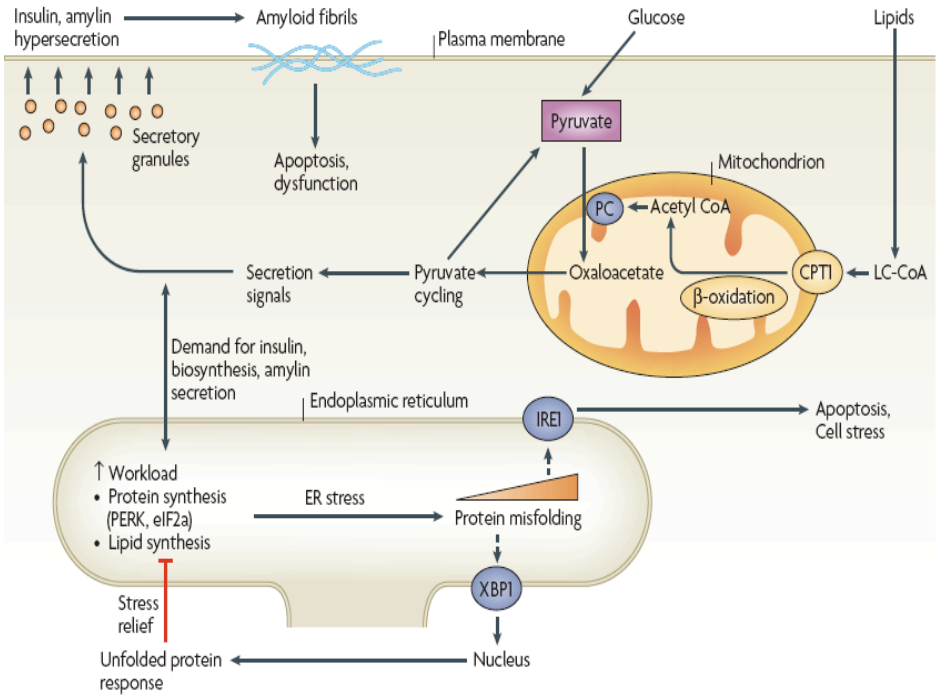

Figure 2.3 Mechanisms of β-cell failure in T2D

The model includes metabolic overload (mitochondria), endoplasmic reticulum (ER) stress and deposition of harmful amyloid fibrils. Overnutrition and increased lipid supply induce enzymes of β-oxidation, such as carnitine palmitoyltransferase-1 (CPT1), resulting in increased acetyl CoA levels, allosteric activation of pyruvate carboxylase (PC) and constitutive upregulation of pyruvate cycling. This leads to basal insulin hypersecretion and loss of the glucose-stimulated increment in pyruvate cycling flux, thereby blunting GSIS. The increased demand for insulin biosynthesis increases workload in the ER, gradually leading to ER stress and increased protein misfolding. ER stress initially relieved by the unfolded protein response (UPR), mediated by the transcription factor XBP1, but over time, the UPR becomes less effective and the deleterious effects of ER stress lead to cell death, mediated by IRE1. Finally, insulin hypersecretion is accompanied by amylin secretion, which in humans can from amyloid fibrils that accumulate at the surface of the β-cell to induce dysfunction and apoptotic death. eIF2a, eukaryotic translation initiation factor-2a; IRE1, inositol-requiring kinase-1; LC-CoA, long-chain acyl CoA; PERK, protein kinase RNA (PKR)-like ER-associated kinase. Adopted from Muoio and Newgard, 2008 [45].

storage forms of this carbohydrate in many organisms [46].

For the cells to utilize glucose, it has to be first phosphorylated into glucose 6-phosphate (G6P). This reaction is catalyzed by a family of enzymes called hexokinases (ATP: D-hexose 6-phosphotransferase, EC 2.7.1.1). Hexokinase is one of the key enzymes of the Embden-Meyerhof glycolytic pathway, which phosphorylates glucose to G6P by using ATP [47], the initial step in metabolism of glucose through most common pathways. In two preliminary studies conducted about 50 years ago, four hexokinases were identified in rat liver as A, B, C and D in the order of elution from DEAE-cellulose columns [48], or I, II, III, IV by the anodal mobility on starch gel electrophoresis [49]. The hexokinase D (IV) is commonly called “GK”. In addition, it was demonstrated that the occurrence of multiple hexokinases in the rat is a general phenomenon not restricted to any single tissue. The proportions of the hexokinase types constituting the total activity of any one tissue are variable, depending on the tissue, age of animal, and nutritional factors [50]. The hexokinase isoenzymes can catalyze the phosphorylation of both α- and β-D-glucose, although with different kinetic constants. Generally, there is a higher limiting rate for phosphorylation of the β-anomers of glucose and mannose, but much higher affinities for the α-anomers [51]. Each hexokinase differs from the others with respect to Michaelis constant for glucose, substrate specificity, immunological reaction, tissue distribution, developmental and adaptive behavior [52]. It is noteworthy that more than four electrophoretically different hexokinases were found in the liver of some mammals [53].

Hexokinases A, B and C are 100 kDa molecules (two 50 kDa domains) thought to have evolved by duplication and fusion of a gene encoding an ancestral 50 kDa hexokinase. Therefore, these isoenzymes display internal sequence repetition, and the N- and C-terminal halves have extensive sequence similarity, both to each other and to other members of the hexokinase family, which includes the 50 kDa mammalian GK and 50 kDa hexokinases found in other organisms [46]. Hexokinases A, B and C share

several properties: (a) low K_m values for glucose (K_m < 1mM); the reactions catalyzed by them are barely affected by changes in the blood-glucose concentration, because they are all largely saturated throughout the physiological range; (b) low K_i values for G6P, i.e. they are strongly and allosterically inhibited by G6P [54]; (c) ability to phosphorylate hexoses like fructose or mannose in addition to glucose; (d) broad tissue distribution.

Hexokinase A is ubiquitously expressed, consistent with the importance of glycolysis in all mammalian tissues. Moreover, it is expressed at particularly high levels in brain, a tissue well known for its virtually total reliance on glycolytic metabolism of glucose to sustain a high rate of energy metabolism. Only the C-terminal domain of hexokinase A is catalytically active, but G6P binds to both domains [55]. On the other hand, hexokinase B is much more limited in its expression, primarily being found in insulin-sensitive tissues such as skeletal muscle and adipose tissue. Unlike hexokinase A, both domains in hexokinase B are catalytically active and sensitive to G6P, and glucose binding to the N-terminal domain decreases the concentration of G6P required to regulate the activity of the C-terminal domain, suggesting that the two halves functionally interact [56, 57]. Hexokinase C expression is relatively low in most tissues, with the highest levels detected in lung, kidney, and liver [58]. Hexokinases A and B contain a conserved 21-amino acid sequence in the N-terminal domain that is predicted to form a hydrophobic α-helix and enables these proteins to bind to mitochondria [59]. In contrast, hexokinase C lacks the hydrophobic N-terminal sequence critical for targeting to mitochondria. It was reported that this isoenzyme was found in the “soluble” fraction of tissue homogenates, indicating a cytoplasmic location [46]. Analysis of promoter governing the transcription of hexokinase A, B and C indicated that they respond to quite different transcription factors, offering the flexibility of hexokinase isoenzymes in expression, in different tissues or in different physiological states [60].

2.3.2 Introduction of GK

In 1958, Cahill *et al.* [61] reported that the rat liver slices incubated in media of increasing glucose concentrations show increased levels of glucose uptake from the medium This observation suggested the existence of a hexokinase with a much higher Michaelis constant in the liver, and led to the discovery of rat liver GK (ATP: D-glucose 6-phosphotransferase, EC 2.7.1.2) [62]. GK differs significantly from other hexokinases. It is half-saturated in the physiological range of glucose concentrations ($K_m \approx 10$ mM). In fact, GK has an affinity for glucose more than 20 times lower than that of hexokinase B, the next ranking hexokinase. Another unique enzyme kinetics of GK is that the steady-state velocity of the GK-catalyzed reaction displays a sigmoid rather than hyperbolic dependence upon increasing glucose concentrations [63], a hallmark of allosteric regulation. This enzyme has cooperative kinetics with its substrates glucose and mannose as manifest by a Hill coefficient of 1.7. Functionally, this positive cooperativity allows the enzyme to have increased sensitivity to fluctuations in blood glucose levels. Further, it is inhibited only weakly and non-allosterically by G6P under physiological conditions, as substantial inhibition requires concentrations above 50 mM [64], consistent with the fact that the liver is able to phosphorylate glucose in the presence of high concentrations of G6P. These kinetic properties enable GK to be highly responsive to glucose levels and to ensure that the glucose metabolic flux is closely tied to the glucose concentration.

It was pointed out that the name “GK” for hexokinase D may be a useful guide to its physiological role; however, it was unsatisfactory as the enzyme is not wholly specific for glucose. In fact, the four hexokinases isoenzymes are similar in sugar specificity, and the difference is that GK is less effective at discriminating between glucose and fructose than some of the other isoenzymes [65]. Mammalian GK accepts a variety of hexose substrates. The specificity of this enzyme demonstrates the following preference of substrates: glucose = mannose > deoxyglucose > fructose = glucosamine [66]. Since its discovery, the enzyme has been considered as a separate entity meriting the

name “GK” and an EC number, which have been considered misleading as they are not specific for glucose [67]. Therefore, some have argued that the name “GK” should be reserved for enzymes that are genuinely specific for glucose, such as those expressed in moulds, bacteria and invertebrate animals [51].

GK is predominantly produced in the liver and the pancreatic β-cells [68, 69], although lower levels have been detected in the hypothalamus, enteroendocrine cells, gonadotropes, and the pituitary gland [70-73].

2.3.3 Metabolic Role of GK

The phosphorylation of glucose by GK is the first rate-limiting step of glucose metabolism in the liver and pancreas. In 1992, two studies using the candidate gene approach reported a genetic linkage between maturity onset diabetes of the young (MODY2) and *Gck* [74, 75], suggesting a key role of GK in glucose metabolism.

All mammalian cells can use glucose as an energy source. In addition, the liver functions as the predominant organ of glucose production, with small contributions by the kidney and the gut, whereas peripheral tissues (skeletal muscle and fat) and the central nervous system remove glucose from the plasma. In the liver, glucose moves freely across the plasma membrane in either direction. At blood glucose levels of approximately 8.33 mM the liver does not take up or supply glucose to the circulation. This level is therefore termed the “steady state” or the “glucostatic level” at which the mechanisms of normal supply and removal of glucose are operating at equal rates. At glucose levels above 8.33 mM glucose removal is greater than supply whereas at levels below 8.33 mM glucose supply is greater than removal, and the fasting blood glucose level in most mammals is about 5 mM [76]. Due to the fluctuations in food consumption and activity level throughout the course of a day, most mammals maintain stable blood glucose levels by continuously adjusting its metabolism in different tissues and organs. To achieve this, the body initiated an intricate system of neural, hormonal and direct nutrient

responses when exposed to stress (exercise or hypoglycemia) or a meal. One of the key factors to this homeostatic control is GK.

GK activity in pancreatic β-cells is responsible for maintaining glucose homeostasis throughout the body. This is achieved through a cascade of cellular events that links the rate of glycolysis to the release of insulin into the bloodstream. This process begins when glucose enters the β-cells via the GLUT family of transporters, where it is phosphorylated by GK. Continuation of the glycolytic pathway produces an increase in the ATP/ADP ratio, which stimulates closure of the ATP-responsive K^+ channels in the β-cell plasma membrane. Upon channel closure, the plasma membrane becomes depolarized allowing an influx of Ca^{2+} into the cell. Combined with intracellular Ca^{2+} pools, these ions promote fusion of insulin-containing secretory granules with the plasma membrane, and in turn, the rise of plasma insulin levels in response to glucose stimulation. The elevated release of insulin causes the translocation of the glucose transporter GLUT4 from intracellular storage vesicles to the plasma membrane and the elevation of glucose uptake and metabolism in the muscle and adipose tissues. Together, these processes act to decrease blood glucose concentrations [77].

2.3.4 Cooperativity of GK with Glucose

Human GK consists of one chain of 448 amino acids forming a monomeric molecule consisting of 13 α helices and 5 β sheets. The chain is folded into two distinct regions, a small and large domain [78]. GK monomer with a single glucose-binding site is active; therefore its cooperative behavior cannot be explained by the classical multisubunit allosteric protein mechanism. A mnemonic model mechanism of positive cooperativity has been proposed for GK in which GK would alternate between low and high affinity conformations involving a slow inter-conversion between the conformational states of glucose-free low affinity GK rapidly evolving to a high affinity state upon binding to glucose. Glucose and an activator modify the space between the large and small globular GK domains, generating a narrow

deep cleft containing the glucose-binding pocket. This ‘closed’ conformation corresponds to the GK high-affinity-active form while in the absence of ligands, GK displays a ‘super-open’ low glucose affinity-catalytically-inactive form [79].

2.3.5 Regulation of *Gck* Expression

2.3.5.1 Alternative Promoters of *Gck*

A defining feature of the *Gck* in mammals is the presence of two alternative promoters. Bedoya *et al*. [80] originally discovered that *Gck* expression control varied greatly from tissue to tissue. The compared GK enzyme activities of liver and pancreatic islets in hyperinsulinemic and hypoglycemic insulinoma-bearing rats and found that hepatic GK was greatly increased and islet GK was drastically reduced when insulin was very high and glucose very low, whereas the opposite was true when insulin was low and glucose high, suggesting organ-specific differential expression control of GK. This idea was substantiated a few years later by the cloning of a single *Gck* and the discovery of two distinct promoters in this gene (an upstream neuroendocrine and a downstream hepatic promoter) [81, 82].

The structure of *Gck* has been characterized in rats, human, and mouse [83-85]. Human *Gck* on chromosome 7p15.3-p15.1 consists of 12 exons that span ~45,169 bp and encode a 448-amino-acid protein, and three tissue-specific isoforms are known [86]. The mouse *Gck* is located on chromosome 11. Two promoters are expressed in a tissue-specific pattern. In 1989, Magnuson and co-workers identified nine exons from rat liver and insulinoma-derived cDNAs. The sequences were essentially identical [81, 83]. The upstream, neuroendocrine promoter, and the adjacent leader exon, termed exon 1β in reference to the β-cells of the islets of Langerhans, drive *Gck* mRNA synthesis in non-hepatic tissues, including pancreatic β cells, central nervous system, lung, thyroid, and gut cells; the downstream hepatic promoter and its associated leader exon, termed exon 1L in reference to the liver, are involved in gene transcription in hepatocytes [63, 87]. The islet-type exon 1β

is located 25-30 kb upstream of the liver-specific exon 1L [88], and the intervening sequence between the two leader exons has yet to be mapped accurately [87].

For the activation of the 1β promoter, glucose seems to be a positive regulator. This may serve the purpose of controlling the insulin secretion. The sequences necessary for expression of the *Gck* in β-cells has been characterized, with 294 bases of 5' flanking sequence required for tissue-specific expression in transgenic mice [70, 89]. Elements in the proximal promoter region have been identified that contribute to transcription in both insulinoma cells and AtT-20 cells. Distal elements may also be involved, although none have been reported [90]. In addition, *Gck* transcription in the islet appears to be largely constitutive.

Although the fragments of the hepatic *Gck* promoter have been mapped and characterized in hepatocytes and hepatoma cells, the identity of all transcription factors regulating *Gck* promoter activity has not been fully elucidated [63]. Insulin and glucagon increases and decreases hepatic *Gck* transcription, respectively [91]. The *cis*-regulatory elements that determine hepatocyte-specific expression and regulation of the hepatic *Gck* isoform are largely undefined as a DNA fragment able to confer both hepatocyte-specific and hormone-regulated expression to a reporter gene has not yet been identified [90]. Transgenic studies have indicated that the sequences necessary for position-independent expression of hepatic *Gck* are located outside a fragment containing sequences from ~ -7.5 kb to + 18 bp (relative to the transcription start site in liver) [85]. Another transgenic study suggested that a DNA fragment of the mouse *Gck*, which spans from - 55 to + 28 kb relative to the liver transcription start site, is expressed and regulated both in the liver and in the islet [90]. In addition, it was reported that a 1 kb fragment of the rat downstream promoter, which contains all hepatocyte-specific DNase I hypersensitive sites that can be detected in a region spanning 35 kb of DNA, is transcriptionally active in primary hepatocytes. However, it failed to respond

to hormones, possibly due to disturbed hormone action in transiently transfected hepatocytes [92].

2.3.5.2 Short-term (Post-translational) Regulation of Hepatic *Gck*

The activity of hepatic GK is controlled post-transcriptionally in the short term by the concentration of its substrate glucose and by a regulatory protein (GKRP), which acts as a competitive inhibitor with respect to glucose. In mammalian species, the effect of this protein is modulated by fructose-6-phosphate (F-6-P), which reinforces the inhibition, and by fructose-1-phosphate (F-1-P), which antagonizes it and releases the GK-GKRP interaction [93]. GK does not have a nuclear localization-signal (NLS) and GKRP, which is mainly located in the hepatocyte nucleus, complexes with GK and sequesters the enzyme in the nucleus in an inactive pool [79, 94]. At low glucose concentration, GKRP is associated with GK in the nucleus. Acute challenge of glucose, fructose, and other precursors of F-1-P induce dissociation of the GK-GKRP complex, and then GK is translocated into cytoplasm, resulting in increased GK activity [95, 96]. The long-term role of GKRP is thought to be a GK stabilizer because GKRP knockout mice showed decreased GK activity and GK protein level [97, 98].

In addition, long-chain acyl-CoAs (C-12 to C-20), such as palmitoyl-CoA, have been shown to inhibit hepatic GK competitively with respect to glucose. Their effect is instantaneous and can be reversed by dilution or upon addition of albumin. Furthermore, they act at concentrations lower than those at which they form micelles and their effect is not mimicked by fatty acids. These data indicate that they do not act as detergents but by binding to an allosteric site on GK [96, 99].

2.3.5.3 Transcriptional Regulation of Hepatic *Gck*

GK activity is largely regulated by the expression of its gene. Many transcriptional factors have been shown to regulate *Gck* promoter activity.

SREBP-1c

SREBP-1c belongs to a family of transcription factors involved in cholesterol and fatty acid metabolism [100]. It was demonstrated that SREBP-1c is a major factor of insulin action on *Gck* expression in cultured rat hepatocytes [101]. In addition, recent work by Kim *et al.* [102] has identified two functional SREBP-1c response elements (SREs) in rat hepatic *Gck* promoter. SREPB-1c can bind to these SREs and activate hepatic *Gck* promoter. However, these notions contrast with the observation that overexpression of SREBP-1c does not affect the level of mRNA for *Gck* in livers of transgenic mice [103], as well as the fact that insulin induces *Gck* transcription at least 4 hours ahead of accumulation of mature SREBP-1c in the nucleus, and knockdown of *Srebp-1* does not affect *Gck* mRNA levels in primary rat hepatocytes [104]. In addition, Hansmannel *et al.* [105] reported that *Gck* mRNA is unresponsive to the liver X receptor (LXR) ligand T0901317 in primary hepatocytes and SREBP-1c does not bind to the hepatic *Gck* promoter. Although the reason for this discrepancy is not readily evident, further studies of the role of SREBP-1c in determining hepatic *Gck* expression may help us to understand how the downstream *Gck* promoter is regulated by insulin and other hormones [106] (see also Section 2.4.6).

FOXO1

FOXO1, a member of the FOXO family of forkhead transcription factors, is phosphorylated by PKB/Akt in the presence of insulin signal and then excluded from the nucleus, resulting in inhibition of the expression of target genes [107]. On the other hand, unphosphorylated form of FOXO1 localizes to the nucleus and interacts with insulin response sequences present within the promoter regions of multiple target genes and activates their transcription. Transfection assays in HepG2 cells suggest that FOXO1 represses the hepatic nuclear factor 4α (HNF4α)-potentiated expression of *Gck* [108]. Transgenic mice expressing constitutively active form of FOXO1 in the liver display reduced expression *Gck* and *Srebp-1c* [109]. A recent study showed

that disruption of *Foxo1* in the liver of mice lacking hepatic *Irs1* and *Irs2* (i.e. disrupted insulin signaling) leads to decreased *Gck* expression [110]. In addition, it was suggested that resveratrol represses *Gck* expression by promoting the recruitment of FOXO1 to the *Gck* promoter and that resveratrol-promoted interaction between FOXO1 and HNF4α contributes to the effects of resveratrol [111]. However, liver-specific deletion of *Foxo1* does not affect hepatic *Gck* expression in mice [112].

Upstream Stimulatory Factor-1 and -2 (USF-1 and -2)

USF is a ubiquitous nuclear protein that was first identified as a factor that bound to an upstream element in, and stimulated transcription from, the adenovirus major late promoter [113]. The USF proteins belong to the class of basic helix-loop-helix zip (bHLHz) factors and act as sequence-specific trans-activators in the form of homodimers or heterodimers, binding DNA in a sequence-specific manner at E-box recognition elements [114]. In the liver, USF1/USF2a heterodimers seem to be the favored DNA-binding species [115]. Proteins of the bHLH gene family bind to sequences of the general type CANNTG, where the central two nucleotides provide for discrimination in binding between different family members [113]. In 1998, Iynedjian [114] identified two protected sequence elements, designated P1 and P2, from *in vitro* DNAse I protection assay using the proximal promoter region and rat liver nuclear protein extract. The sequence of the P2 element contains the core motif CACGTG, which serves as a binding site for a substantial number of transcriptional activators including USF [113]. Co-transfection of an expression plasmid coding for USF1 activated hepatic *Gck* promoter activity, whereas expression of a truncated form of USF1 lacking the transcription activation domain and the basic region decreased reporter activity by a dominant-negative effect [114]. Consistently, a recent study showed that hypoxia inducible factor 1 alpha (HIF-1α) and USF2a are able to bind to the same site and activate hepatic *Gck* promoter in response to venous pO_2. In

addition, HIF-1α, but not USF1, can synergize with HNF4 to activate the hepatic *Gck* promoter [116].

Hypoxia-Inducible Factor-1α (HIF-1α)

HIF-1α is ubiquitously expressed in human and mouse tissues and has a general role in multiple physiological responses to hypoxia, such as erythropoiesis and glycolysis [117]. Transient transfection in primary rat hepatocytes has revealed that HIF-1α can affect promoter activity of hepatic *Gck* by binding to the -87/-80 bp region of *Gck* promoter [116]. Both insulin and hypoxia up-regulate hepatic *Gck* expression by increasing HIF-1α level and its binding activity [118]. It was also reported that HNF4α and coactivator p300 could enhance the HIF-1α-activated hepatic *Gck* transcription [119].

Peroxisome Proliferation-Activated Receptor γ (PPARγ)

PPARγ belongs to the superfamily of nuclear receptors and acts as a transcriptional factor that dominates the regulation of adipose differentiation, lipid storage, and of genes involved in energy storage and utilization. Upon ligand binding, PPARγ heterodimerizes with RXRα, binds to the PPAR response element (PPRE), and activates target gene transcription. It was suggested that a PPRE is located in the -116/ -114 bp region of the rat hepatic *Gck* promoter and that thiazolidinedionse (TZDs), synthetic ligands of PPARγ, can induce *Gck* expresson in primary hepatocytes [120]. A recent study reported that liver X receptor α (LXRα)-dependent activation of hepatic *Gck* in Alexander cells is partly through activation of this PPRE [121]. In addition, over-expression of PPARγ1 in PPARα$^{-/-}$ mouse liver notably increased *Gck* expression [122].

HNF4α

HNF4α is a member of the nuclear receptor superfamily of transcription factors and binds as a homodimer to a relatively degenerate consensus DNA sequence consisting of two direct repeats separated by one or two

nucleotides [123]. It was first identified by its interaction with a *cis*-regulatory sequence of liver specific gene promoters [124]. Three different genes coding for three different isoforms have been identified, HNF4α and HNF4γ in mammals, drosophila, and xenopus, and HNF4β in xenopus. The expression of HNF4α is restricted to liver, kidney, small intestine, colon, pancreas and testis. HNF4γ is expressed in the same tissues, except for liver [124, 125]. HNF4α is a central regulator of gene expression in cell types that play a critical role in metabolic homeostasis, including hepatocytes, enterocytes, and pancreatic β cells [126]. Mutations in human *Hnf4α* are associated with MODY1, an autosomal dominant genetic disorder that is characterized by early onset T2D [127]. MODY1 patients are hyperglycemic and hypoinsulinemic, have reduced levels of circulating lipids, and display defects in the expression of genes involved in glucose and lipid metabolism [128, 129]. It was showed that *Gck* expression was activated by HNF4α via the sequence -52/-39 of the *Gck* promoter. In addition, HNF4α might act as a regulatory factor mediating the perivenous zonated *Gck* expression [130] (see also Section 2.5.3).

2.4 SREBPs in the Regulation of Lipid Homeostasis

2.4.1. Introduction

SREBPs belong to the basic helix-loop-helix-leucine zipper (bHLH-Zip) family of transcription factors and coordinate the synthesis of fatty acids and cholesterol. SREBPs are synthesized as inactive precursors anchored in the endoplasmic reticulum (ER) and nuclear membrane [100, 131]. Each SREBP precursor is consisting of three domains: an NH_2-terminal domain of about 480 amino acids that contains the bHLH-Zip region for binding DNA; two hydrophobic transmembrane-spanning segments interrupted by a short loop of about 30 amino acids that projects into the lumen of the ER; and a COOH-terminal domain of about 590 amino acids that performs the essential regulatory functions [131]. In order to enter the nucleus and influence transcription, the NH_2-terminal domain of SREBPs must be released from the

membrane, which is accomplished by the coordination of four proteins: SREBP cleavage-activating protein (SCAP), insulin-induced genes (Insigs), Site-1 protease (S1P), and Site-2 protease (S2P). Under high cholesterol conditions, binding of cholesterol to the sterol-sensing domain induces a conformational change that results in SCAP binding to ER retention protein Insigs, preventing vesicular trafficking of SREBPs from the ER to the Golgi apparatus. In cholesterol-depleted cells, however, SCAP escorts SREBPs from the ER to the Golgi apparatus, where the two-step proteolytic cascade takes place [132, 133]. S1P, a membrane-bound serine protease, cleaves the SREBP in the lumen loop between the two membrane-spanning segments and the NH_2-terminal bHLH-Zip domain is released from the membrane upon a second cleavage mediated by the membrane-bound zinc metalloproteinase S2P [131].

The mammalian genome encodes three SREBP isoforms: SREBP-1a, SREBP-1c, and SREBP-2. The first two isoforms are derived from a single gene on human chromosome 17p11.2 through the use of alternative splicing, whereas SREBP-2 is encoded by a gene on human chromosome 22q13. While SREBP-1a is a potent activator of all SREBP-responsive genes, SREBP-1c preferentially enhances transcription of genes involved in fatty acid synthesis, but not cholesterol synthesis. SREBP-2 preferentially activates cholesterol synthesis [131]. It has been shown that SREBP-1a and SREBP-2 are preferentially expressed in most culture cell lines, whereas SREBP-1c and SREBP-2 predominate in the liver and most other intact tissues [134].

2.4.2 SREBP-1c in Liver

Transgenic mice expressing the NH_2-terminal fragment of SREBP-1c display increased hepatic expression of genes encoding proteins involved in fatty acid synthesis (acetyl CoA carboxylase, fatty acid synthase, and stearoyl-CoA desaturase-1), but not in cholesterol uptake (LDL receptor) and synthesis (HMG CoA synthase, HMG CoA reductase, and squalene synthase) [135]. Similarly, the liver express a mutant version of SCAP that is resistant to

feedback suppression by sterols, the amounts of nSREBP-1 and -2 rise and there is a concomitant overproduction of cholesterol and fatty acids, leading to marked engorgement of the liver with cholesteryl esters and triglycerides [136]. In contrast, deficiency of SCAP [137] and S1P [138] in mouse liver result in reduced rates of cholesterol and fatty acid synthesis due to declined nuclear SREBPs.

Germline knockout of SREBP-1 [139] or SREBP-1c [140] in mouse lead to a compensatory increase in hepatic expression of SREBP-2 and therefore increased mRNA levels for cholesterol biosynthetic enzymes. Germline disruption of SREBP-2 [139] or S1P [138, 141] causes embryonic lethality in mice.

2.4.3 SREBP-1c in β Cells

SREBP-1c plays an important role in β-cell lipotoxicity. In pancreatic MIN6 β-cells, SREBP-1c is required for glucose-stimulated expression of acetyl-CoA carboxylase expression, a key lipogenic enzyme. On the other hand, over-expression of SREBP-1c decreases glucose-stimulated insulin release, indicating a critical role of SREBP-1c in promoting β-cell dysfunction [142]. Similarly, in another insulin secreting cell line INS-1, over-expression of nuclear active form of SREBP-1c using tet-on inducible system or adenovirus induces massive accumulation of lipid droplets and blunted nutrient-stimulated insulin secretion [143, 144]. In addition, adenovirus-mediated over-expression of SREBP-1c in rat islets induces genes involved in cholesterol, fatty acid, and eicosanoid synthesis [145].

2.4.4 SREBP-1c in Adipose Tissue

In addition to liver, adipose tissue is another organ that produces an abundance of SREBP-1c relative to -1a. It has been shown that the mRNA level of *Srebp-1c* exceeds that of *Srebp-1a* by 3-fold [134].

The implication of SREBP-1c in adipose differentiation was first demonstrated by Spiegelman and coworkers [146], who identified SREBP-1c

as an activator of fatty acid synthesis and differentiation of mouse 3T3-L1 pre-adipocytes into mature adipocyte in tissue culture. SREBP-1c therefore was originally named adipocyte determination- and differentiation-dependent factor 1 (ADD1). On the other hand, ectopic expression of a dominant-negative SREBP-1c in 3T3-L1 preadipocyte cell line strongly inhibits the adipocyte differentiation and suppresses the expression of adipocyte-specific genes [147].

Stimulation of the *Srebp-1c* with LXR agonist has negligible effects on the expression of lipogenic enzymes in adipocytes, despite a concomitant increase in nuclear active SREBP-1c, indicating that regulation of lipogenic genes is independent of SREBP-1c in mouse adipocytes [148]. Disruption of *Srebp-1* has little effect on the dynamic changes (*i.e.* fasting-refeeding) of lipogenic gene expression in adipose tissue [149]. In transgenic mice, over-expression of n*Srebp-1c* in adipose tissue under the control of the adipocyte-specific aP2 enhancer/promoter resulted in disruption of the differentiation processes of adipocytes, insulin resistance, hyperglycemia, fatty liver from birth and elevated plasma triglyceride levels later in life [150].

It has been shown that the mRNA expression of *Srebp-1c* in adipose tissue is reduced dramatically upon fasting and elevated upon refeeding [151]. Similar observations were obtained from healthy individuals and insulin resistant non-diabetic patients, in which *Srebp-1c* mRNA levels were induced by insulin [152]. These observations suggest that insulin regulates the mRNA expression of *Srebp-1c* both in mice and human.

2.4.5 SREBP-1c in Muscle

It has been suggested that skeletal muscle *Srebp-1c* expression is regulated by nutritional status in a fashion similar to that observed in liver and adipose tissue [153]. Insulin induces the expression of *Srebp-1c* in cultures of adult rat skeletal muscle cells [154] and in human [152]. Leptin suppresses refeeding effects on *Srebp-1c* mRNA levels in rats [153].

2.4.6 Insulin-stimulated Expression of SREBP-1c in the Liver

The hepatic SREBP-1c expression is reduced during fasting but increases markedly when animals are refed a high carbohydrate diet. In contrast, such manipulations induce only minor effects on the expression of the other SREBP isoforms [155]. In addition, rats treated with STZ display dramatically reduced mRNA level of *Srebp-1c*, and insulin treatment restores both mRNA and protein levels of SREBP-1c. However, these treatments have little effect on the mRNA expression of *Srebp-1a* and *Srebp-2*, indicating the *in vivo* role of insulin in regulating *Srebp-1c* transcription [156]. In primary hepatocytes that transcription of SREBP-1c is induced by insulin and suppressed by glucagon via induction of cellular cyclic AMP level [157].

Studies using primary rat hepatocytes revealed that insulin activates the transcription of *Srebp-1c* and the synthesis of the precursor form of SREBP-1c, through the PI3K/Akt pathway [158, 159]. PKCλ, which acts downstream of PI3K, also plays a critical role in mediating insulin-induced SREBP-1c expression in liver [160, 161].

SREBP-1c has been suggested to regulate the expression of genes involved in glucose metabolism. A dominant active form of SREBP-1c in primary hepatocytes induces *Gck* expression to similar extent as insulin treatment [101]. This led to the conclusion that SREBP-1c is the mediator of insulin-stimulated *Gck* expression in the liver and hepatocytes. However, this view has been challenged by the several studies using different models. In the study using liver-specific *Srebp-1c* knockout mice, the hepatic *Gck* expression responded normally to the cycle of fasting and refeeding [140]. The knockdown of *Srebp-1c* by small interfering RNA in primary hepatocytes did not result in any change in insulin-induced *Gck* expression [104]. In our time course study, insulin-induced *Gck* expression peaked at three hours after the stimulation, a time point before significant induction of SREBP-1c protein. The insulin-induced *Srebp-1c* mRNA expression peaked at 9 h, which is not enough to mediate the insulin-induced *Gck* expression [12].

The insulin-induced *Srebp-1c* expression in primary hepatocytes has also

been considered to mediate insulin-suppressed *Pck1* expression. In that study, overexpression of constitutive active SREBP-1c in hepatocytes caused reduction of *Pck1*, which is an insulin-suppressed gene. However, our time course study excluded the possibility of this mechanism to mediate short-term regulation of *Pck1* expression by insulin [12].

2.4.7 Role of LXR in the Regulation of *Srebp-1c* Expression

LXRα and β are members of the nuclear receptor superfamily that heterodimerize with RXR [162]. LXRα is highly expressed in liver, intestine, kidney, and adipose, whereas LXRβ is expressed more ubiquitously with particularly high levels in the developing brain [163, 164]. Oxysterol, oxidized derivatives of cholesterol, are endogenous ligands for LXRs [165]. Activated LXRs have been shown to regulate expression of genes important in cholesterol metabolism as well as fatty acid synthesis [166].

LXRs play an important role in the regulation of SREBP-1c expression. It was demonstrated dietary cholesterol and synthetic agonists for LXRs and RXR induced SREBP-1c expression through the LXR element (LXRE), without affecting expression of SREBP-1a and -2. Hepatic SREBP-1c expression is significantly reduced in mice deficient in both LXRα and LXRβ; however, deficiency of only LXRα slightly reduces SREBP-1c mRNA while there is no effect when only LXRβ in lacking [167]. LXR also plays a central role in mediating the effect of insulin on *Srepb-1c* transcription. Chen *et al.* [168] used reporter constructs to show that mutation of the LXREs in *Srebp-1c* promoter significantly reduced insulin-mediated activation of *Srebp-1c* transcription. In addition, high-fat feeding stimulates lipogenesis, with an increase in SREBP-1c expression, possibly through LXR [169].

The primary role of LXR is thought to be in the control and protection of cells from cholesterol overload [170]. Thus the reason why LXR would trigger a parallel increase in fatty acid synthesis via transcriptional regulation of *Srebp-1c* remains unclear. One hypothesis is that the blood transport and cell storage of excess cholesterol requires the formation of cholesteryl ester. This

increases the demand for oleate production, which is indeed stimulated upon over-expression of *Srebp-1c* [170].

SREBP-1c also positively regulates its own production in a feed-forward loop that ensures higher SREBP-1c production when the levels of the mature nuclear form are elevated [171].

It was reported that high levels of polyunsaturated fatty acids (PUFA) repress SREBP-1c expression through several possible mechanisms. First, PUFA suppresses SREBP-1c expression by inhibiting the LXR binding to the promoter [172, 173]. Second, PUFA may suppress SREBP-1c expression by accelerating the decay of *Srebp-1c* transcripts as demonstrated in rat hepatocytes [174].

2.5 Nuclear Receptors (NRs) in the Regulation of Glucose and Lipid Metabolism

2.5.1 Introduction of NRs

NRs represent a family of transcription factors that mediate a complex array of extracellular signals into transcriptional responses in a ligand-dependent manner [175]. This family includes receptors for endocrine steroids, fat-soluble vitamins A and D, thyroid hormone, and a large number of “orphan” NRs, whose ligands, target genes, and physiological functions were initially unknown [162, 175-177]. Like other transcriptional regulators, NRs exhibit a modular structure including six regions, A, B, C, D, E and F, with different degrees of evolutionary conservation. The variable N-terminal A/B region contains an autonomous transcriptional activation function 1 (AF-1). The highly conserved region C that corresponds to the core of the DNA-binding domain (DBD) is responsible for specific binding to cognate response elements. Region E contains the ligand-binding domain (LBD), a ligand-dependent transcriptional activation function 2 (AF-2) and a dimerization interface. A variable region D functions as a flexible hinge between the DBD and LBD and contains the nuclear localization signal. Some but not all NRs contain a variable C-terminal region F of unknown function [162, 178-180].

Upon ligands binding, NRs undergo a conformational change that coordinately dissociates co-repressors and facilitates recruitment of coactivators to enable transcriptional activation. NRs control the expression of a network of genes with key roles in glucose and lipid metabolism [162, 181].

There are 48 known NRs in the human genome [162, 182], four of which are focused in this dissertation: retinoic acid receptor α (RARα), RXRα, HNF4α, and chicken ovalbumin upstream promoter-transcription factors II (COUP-TFII).

2.5.2 RARα and RXRα

RA, the biologically active metabolite of VA, exerts its action through two families of NRs, the RARs (α, β, γ) and the RXRs (α, β, γ), which bind as RAR/RXR heterodimers to response elements (RARE) located in the promoters of RA-target genes. The binding of RA to RAR/RXR induces a conformational change that is followed by the replacement of co-repressor with co-activator complexes (see also Section 2.8.6). Most of the RAREs are composed of two hexameric motifs, 5'-(A/G)G(G/T)TCA-3', arranged as palindromes, direct repeats (DRs), or inverted repeats (IRs). The most frequent DRs with 1, 2, or 5 nucleotide spacing are termed DR1, DR2, and DR5 elements, respectively. DR1 elements exhibit different polarity of binding of the liganded RAR subunit compared to the DR2 and DR5 elements. For DR1 elements, an upstream half site is recognized by a RAR and this type of RARE-bound complex acts as a transcriptional repressor. RXRs can also bind as homodimers to DR1 elements and respond to 9-cis RA. In contrast, for DR2/DR5 elements, a RAR occupies the downstream halves of these RAREs and the NR complex functions as transcriptional activator. Additional arrangement of two or three hexameric motifs with variable spacing have also been identified [183].

Unlike the steroid receptors that function as homodimers, a lot of NRs, other than RARs, require RXRs for high affinity binding to their cognate responsive elements. These include thyroid hormone receptors (TRs), vitamin

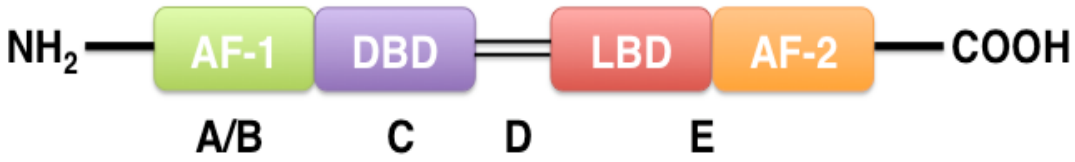

Figure 2.4 Schematic structure of a typical NR

See text for details.

D3 receptors (VDRs), and peroxisome proliferation activated receptors (PPARs) [178, 184]. In fact, RXRs are common partners in at least 11 distinct signaling pathways. However, the activation state of RXR varies among these heterodimers and seems to depend on the nature of its partner [185]. For example, in the case of RAR/RXR heterodimer, it is believed that both partners of the heterodimer can be transcriptionally active. However, the liganded RXR is not active unless its RAR partner is itself liganded [186]. In a PPAR/RXR heterodimer, both PPAR and RXR can bind their cognate ligands and activate transcription, with the binding of both ligands resulting in synergistic activation. Heterodimers in this case are referred to as permissive. Similarly, LXR/RXR heterodimer retains *9-cis* RA responsiveness, further supporting that the view that RXR can be an active partner [176]. In contrast, the TR/RXR and VDR/RXR heterodimers are thought to be nonpermissive, as they are activated by the TR ligand triiodothyronine (T3) and VDR ligand 1,25-dihydroxy-VD3 (calcitriol), respectively, but not by RXR-specific ligands. It is generally believed that in a nonpermissive heterodimer, RXR is incapable of ligand binding and thus is often referred to as a silent partner. However, recent data indicated that RXR was able to bind ligand and lead to dissociation of corepressors from TR, thus modulating heterodimer activity [187].

Genetic studies have established RXRα/RAR heterodimers as the main functional units transducing RA signals during development, and specific heterodimers (RXRα/RARα, RXRα/RARβ, and RXRα/RARγ) are involved in given developmental processes [188].

2.5.3 HNF4α

HNF4α (NR2A1) is a highly conserved member of the NR superfamily of transcriptional factors. Expression of HNF4α gene (*Hnf4a*) is driven by two distinct promoters, the P1 promoter that drives expression of splice variants HNF4α1-6 in the liver, kidney, and intestine/colon and the P2 promoter that

drives expression of splice variants HNF4α7-9 in the intestine/colon, stomach, and β-cells of the pancreas [189].

HNF4α binds DNA as a homodimer and generally acts as a positive transcriptional regulator of many hepatocyte genes. It was found in 1996 that mutations in human HNF4α gene caused MODY1, an autosomal dominant genetic disorder that is characterized by early onset T2D [190], indicating a critical role of HNF4α in metabolic homeostasis. Liver-specific knockout of *Hnf4a* in mouse resulted in accumulated lipid in the liver, reduced serum cholesterol and TG levels, and increased serum bile acid concentrations [191]. In addition, pancreatic β-cell-specific *Hnf4a* knockout mice have impaired glucose-stimulated insulin secretion [192]. It was reported recently that drosophila HNF4 (dHNF4), the single ancestral ortholog of HNF4α, regulates lipid mobilization and β-oxidation [193]. *dHnf4* null mutant larvae were unable to efficiency mobilize stored fat for energy under starvation, consistent with reduced expression of genes that control lipid catabolism and β-oxidation. In addition, *in vitro* studies have established that HNF4α is important in controlling many of the functional properties of developed and mature pancreatic β-cells. For example, HNF4α activates the insulin gene expression through indirect and direct mechanisms [194]. HNF4α also regulates the expression of other pancreatic β-cell genes implicated in glucose metabolism and nutrient-induced insulin secretion, including glucose transporter-2 and L-pyruvate kinase [195]. Moreover, it has been shown that HNF4α can activate hepatic *Gck* expression by binding to its promoter [108, 111, 130].

HNF4α, although initially believed to be an orphan receptor, its activity can be modulated by fatty acyl-coenzyme A (CoA) thioesters, and also by protein kinase A-mediated phosphorylation. This suggests that HNF4α may be responsive to dietary signals and important in the control of metabolic status [191]. Consistently, fatty acids released from triglycerides can activate HNF4 in fasted drosophila, which in turn drives fatty acid oxidation for energy production [193].

A relationship between retinoids and HNF4 has been indicated in controlling the hepatocyte phenotype. It was found that RA-mediated down-regulation of α-Fetoprotein gene was dependent on the inhibition of HNF1 and HNF4 in Hep3B cells [196]. Since there is no RARE at the HNF4 gene promoter, the mechanism for RA-mediated inhibition of HNF4 remains to be investigated. On the other hand, HNF4α regulates retinoid metabolism by activating the transcription of CRBPII gene [197]. Furthermore, it is suggested that HNF4α and RXRα compete for occupancy of the same site in cytokine erythropoietin gene promoter and sequentially regulating its expression during embryogenesis [198].

2.5.4 COUP-TFII

COUP-TFs are among the most studied orphan NRs with no physiological ligand. In humans, COUP-TFs consist of at least three members, COUP-TFI (also called NR2F1 or ErbA-related protein-3 [EAR3]), COUP-TFII (also called NR2F2 or apolipoprotein-AI regulatory protein-1 [ARP1]), and the more distant COUP-TFIII (also called NR2F6 or EAR2 [199]. COUP-TFs are the most evolutionarily conserved NRs among all species, with the LBDs of COUP-TFI or II being identical in vertebrates [200], suggesting their functional importance. In fact, deletion of either COUP-TFI or COUP-TFII in the mouse is lethal [201]. However, COUP-TFII is required earlier in development than COUP-TFI [202].

COUP-TFII is implicated in a variety of biological processes, including development, cellular differentiation, growth, and metabolic homeostasis [203]. *In vitro* studies have shown that COUP-TFII regulates insulin gene expression in pancreatic β-cells and several genes involved in glucose and lipid metabolism [204, 205]. Gain-of-function and loss-of-function studies have established COUP-TFII as a potent repressor of adipogenesis by suppressing the expression of a number of pro-adipogenic factors, including SREBP-1c, PPARγ1, PPARγ2, and C/EBPα [206]. Since global COUP-TFII deficiency is lethal to embryos, conditional knockout mice had been generated to study the

physiological roles of COUP-TFII in nutrient metabolism. It has been shown that heterozygous mutant mice with COUP-TFII deleted from pancreatic β cells have impaired glucose sensitivity and abnormal insulin secretion [207]. In addition, COUP-TFII plays an important role in regulating white adipose tissue development and energy metabolism, as shown in COUP-TFII heterozygous knockout mice that have improved glucose homeostasis and increased EE [208].

Despite that COUP-TFII can activate transcription in certain cell types and promoter contexts, it act primarily as repressors of ligand-mediated NR signaling pathways via both protein-protein and protein-DNA interactions. For example, COUP-TFII binds promiscuously to hormone response elements (HREs) recognized by other NRs, thereby competing with them for their target sites. It has been shown that COUP-TFII can bind DNA by a Zn finger DNA binding domain on a variety of HREs that contain direct or inverted imperfect AGGTCA repeats with various spacings [209]. COUP-TFII can also titrate the common heterodimerization partner, RXR, which is required for high affinity DNA binding of most members of the thyroid hormone/RAR subfamily. In addition to these indirect mechanisms of repression, COUP-TFII can also actively silence basal and activated transcription, likely through direct interaction with TFIIB or other general transcription factors. COUP-TFII therefore antagonizes cellular responses to multiple hormone signaling pathways and can have profound effects on metabolic homeostasis [210].

It has long been clear that COUP-TFs are involved in the modulation of RAR- and RXR-mediated responses to retinoids during embryogenesis [199]. Recently, COUP-TFII was identified as low-affinity RA receptor. High concentration of RA is able to activate COUP-TFII and release it from the autorepressed conformation [200]. These observations suggest a linkage between RA and COUP-TFII signaling pathways.

2.6 Vitamin A

2.6.1 Brief History of Vitamin A

The importance of a substance present in certain foods for the treatment of night blindness was already known in ancient Egypt. The Greek physician Hippocrates (460- 327 B.C.) recommended the intake of “raw beef liver, soaked in honey, once or twice by mouth” for the treatment of “nyctalopia”, or the total inability to see in darkness [211]. In 1881, N. Lunin in Russia reported that mice could not survive on a purified diet of fats, carbohydrates, proteins, and salts alone, but survival was more likely when whole milk was added [212]. Thirty years later, F. G. Hopkins [213] found that animals fed a purified diet of casein, starch, sugar, lard and salts failed to grow, whereas the addition of a small supplement of whole milk equivalent to 2-4% of total calories restored normal growth, consistent with Lunin’s observations. Hopkins postulated the existence of unknown substances in the milk that were necessary for life and growth. He called these substances “accessory food factors”. In 1913, McCollum ultimately showed that there was a fat-soluble growth factor in butter fat that could be transferred from one fat to another and this factor was essential for growth and survival of rats. It was named “fat-soluble factor A” as opposed to other accessory dietary factors, called “water-soluble B” [211]. The factor was finally named vitamin A (VA) in 1920 [214]. In 1931, Karrer and co-workers determined the structure of retinol, and soon thereafter the structure of β-carotene. In 1937, retinol was first crystallized from fish liver oil, from which the first crystalline esters were also isolated five years later. In 1946, Van Dorp and Arens synthesized retinoic acid and in 1947 the Isler group reported a commercially feasible synthesis of retinol. In 1950, Karrer and Eugster synthesized β-carotene. Synthetic retinol serves as the precursor for the retinyl esters (REs) used widely in VA supplements and in fortified food. These early developments have been summarized by Wolf [211].

2.6.2 Nomenclature

Retinoids are a class of compounds derived from four isoprenoid units joined in a head-to-tail manner to produce a monocyclic parent compound containing five carbon-carbon double bonds and a functional group at the acyclic terminus, with or without biological activity. The term “VA” groups any retinoid with the biologic activity of all-*trans* retinol (hereinafter referred to as retinol), the parent retinoid compound [215]. It includes both preformed VA, that is, biologically active derivatives of retinol, and provitamin A carotenoids [216].

VA exists both in plants and animals. More than 600 carotenoids have been identified from plants. However, only 50 serve as precursors of VA in humans, which includes β-carotene, the best-known carotenoid [216]. Animal products contain VA predominantly in the form of REs, an esterified product of retinol and a fatty acid, but also as retinol and, in small amounts, as provitamin A carotenoids originating from plants consumed by the animals. In most animal tissues, the predominant retinoid is retinyl palmitate, but other fatty acid esters, such as retinyl oleate and retinyl stearate, are also found. Retinol moiety in most of these metabolites is in the all-*trans* configuration. The 11-*cis* aldehyde form, 11-*cis* retinal, is the chromophore in the retina of the eye, and several acid forms such as the all-*trans*, 3, 4-didehydro- and 9-*cis* retinoic acid, are active metabolites of retinol found in most if not all tissues [217].

2.6.3 Metabolism of VA

2.6.3.1 Absorption of Retinol

The uptake of VA by the absorptive cells of the small intestine is the necessary first step in its utilization by the organism. Dietary VA is available mainly in the form of provitamin A precursor compounds from plants and of preformed VA, REs from animal tissues. All these compounds are lipid soluble and therefore require normal fat digestion and absorption for their absorption.

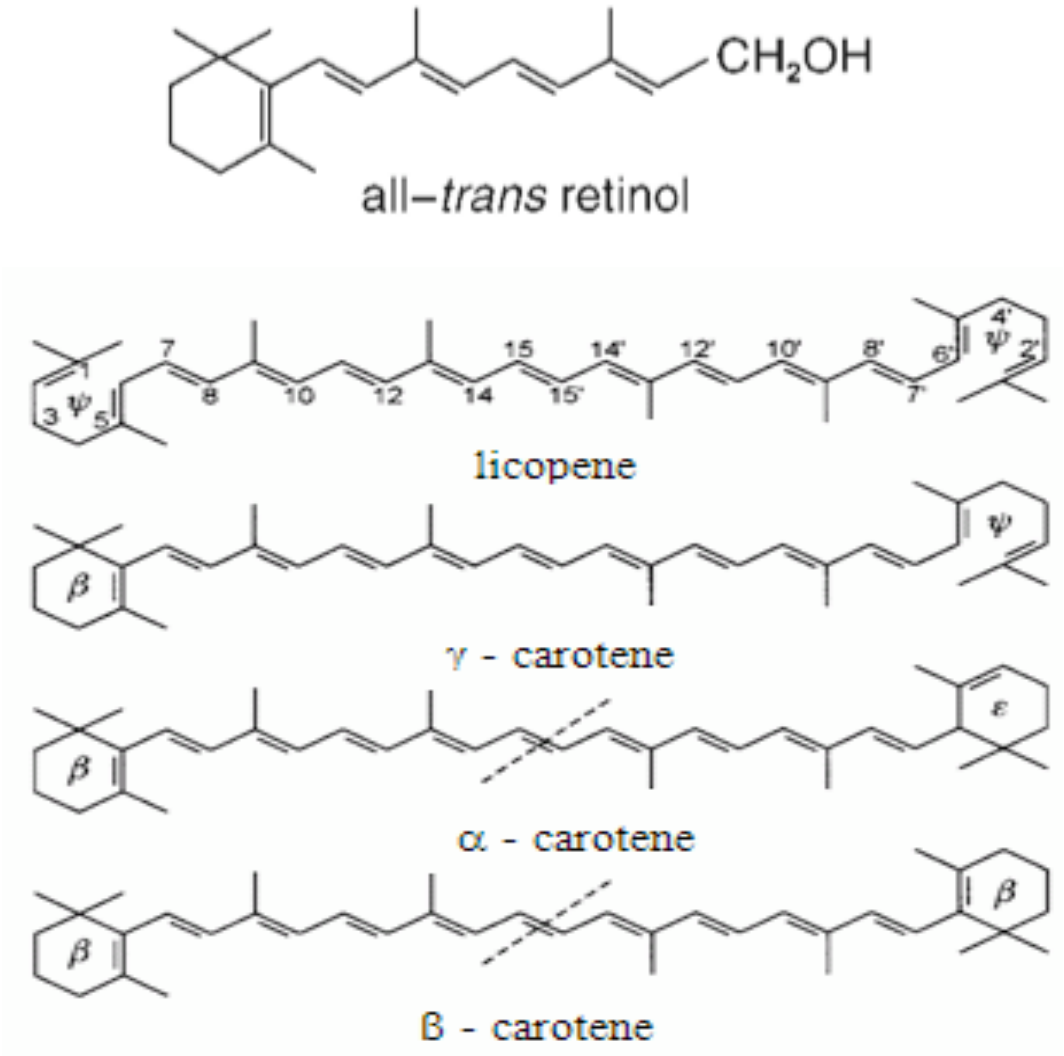

Figure 2.5 Chemical structure of all-*trans* retinol and some carotenoids [218].

Carotenoids are organic hydrocarbon-based pigments naturally occurring in plants, some bacteria, and algae. There are two types of carotenoids: xanthophylls (oxygen containing) and carotenes (without oxygen). The carotenes are vitamin A-related substances produced by plants but cannot be synthesized by animals. There are several forms of the carotenes but only 4 forms (α, β, γ, and β-cryptoxanthin) have VA activity in humans. The α and β forms are the 2 primary forms found in plants [219].

2.6.3.2 Absorption of Carotenoids

It is considered that 5- 50% of carotenoids, dependent on the quantity of dietary fat, can be absorbed by passive diffusion. Absorption efficiency is reduced with increased carotenoid intake. Carotenoids may be converted to VA by central cleavage to yield one or two molecules of retinal, which is catalyzed by a 15, 15'-dioxygenase enzyme that is found in the intestine mucosa, liver and other tissues. Retinal is then reduced to retinol by a aldehyde reductase in intestinal cells [220]. Eccentric cleavage of carotenoids, which is catalyzed by β, β-carotene-9' 10'-dioxygenase, yields β-apo-carotenals with different chain lengths, which then may be shortened by β-oxidation to retinol. In addition, there is evidence showing that retinoic acid may be formed directly from such carotenoids as β-carotene by a still undefined pathway [221]. However, the exact mechanism of conversion of carotenoids to retinol is still controversial.

2.6.3.3 Absorption of REs

In contrast to carotenoids, REs from the diet are hydrolyzed into retinol and fatty acid in the intestinal lumen, which can be catalyzed by several enzymes, including pancreatic lipase, pancreatic carboxyl ester lipase, and one or more RE hydrolases associated with the brush border membranes [222]. Free retinol is incorporated into lipid micelles in the gut lumen (i.e. emulsification) and taken up by absorptive epithelial cells of the intestine. It has been shown that retinol is absorbed by facilitated diffusion at physiological concentrations (up to 150 nM) and by passive diffusion at pharmacological levels (450-2,700 nM) [223]. A specific transporter for retinol may also play a role in the absorption of free retinol [224].

2.6.3.4 Re-esterification of Retinol

Nearly all of the newly absorbed retinol, independent of its source, is transported from enterocytes via the lymphatic route to the general circulation in the form of REs associated with chylomicrons. It has been considered that

more than 80% of the retinol that leaves the intestine in chylomicrons is esterified, in both rats and humans [225]. Therefore, the intestinal esterification of retinol is a key step in VA metabolism [226].

Within the intestinal mucosa, all retinol is re-esterified with long-chain fatty acids (primarily palmitic, with smaller amounts of stearic, oleic and linoleic acids) through the action of two enzymes: lecithin: retinol acyltransferase (LRAT) that interacts directly with the retinol-CRBPII complex and acyl CoA: retinol acyltransferase (ARAT) that interacts with free retinol [226-232]. LRAT utilizes the fatty acid in the *sn*-1 position of membrane-associated phosphatidyl choline as the source of fatty acid for esterification [228, 233], whereas the activity of ARAT requires fatty acyl-CoA [234]. The K_m of the LRAT reaction for retinol is lower than that of the ARAT reaction, whereas the capacity (V_{max}) of the ARAT reaction is substantially greater than that of the LRAT reaction [235]. Although the V_{max} of LRAT is low, it is considered to be sufficient to esterify retinol in the range of standard concentrations of retinol [236]. It has been suggested that LRAT is the major contribution to RE synthesis under normal physiological conditions when cell CRBP-II content exceeds the retinol, and ARAT esterifies excess retinol when large doses are absorbed and CRBP-II are saturated [225]. However, in tissues that do not synthesize CRBP, such as mammary gland, ARAT is the only physiologically active enzyme that esterifies retinol [235].

2.6.3.5 Metabolism of REs in Chylomicrons

Although most of the absorbed VA is secreted into lymph as chylomicron REs, a significant amount is also secreted into portal circulation as unesterified retinol [237]. The portal absorption is likely to be important for pathological conditions that affect secretion of chylomicrons, such as abetalipoproteinemia [238] .

Chylomicrons are exclusively synthesized in the intestine to transport dietary fat and fat-soluble vitamins into the blood. Chylomicrons consist of a hydrophobic core that contains TG, cholesteryl esters, REs, other fat-soluble

vitamins, and about 30% of the free cholesterol, whereas the surface membrane consists of a monolayer of phospholipid, apoproteins, free cholesterol, and some TG [239, 240]. In the general circulation, chylomicron remnants are formed after the lipoprotein lipase (LPL)-mediated hydrolysis of triglyceride and the transfer of apolipoproteins, phospholipids, and free cholesterol to other lipoproteins. The formed chylomicron remnant, comprising only 4% of the original chylomicron mass, is depleted in TG and more enriched in cholesteryl esters, phospholipids, and proteins [240]. However, essentially all chylomicron REs are associated with the particle during the conversion to chylomicron remnants, which are transported either to extrahepatic tissues or to the liver.

2.6.4 Uptake of VA

2.6.4.1 Extrahepatic Clearance of REs in Chylomicron Remnants (CRs)

Goodman *et al.* injected chylomicrons containing newly absorbed labeled VA intravenously into normal intact rats and observed the tissue distribution of radioactivity for several days. In so doing, they found that in rats approximately 25% of postprandial REs could be taken up by kidney, spleen, heart, adipose tissue, lung, skeletal muscle, and adrenals [241]. Other important extrahepatic sites for uptake of REs include bone marrow [242], myeloid leukemic cells [243] and leukocytes [244]. It has been shown that the level of LPL expression in skeletal muscle, adipose tissue and heart directly correlated with the amount of chylomicron VA taken up by the tissues, indicating a critical role of LPL in facilitating uptake of postprandial VA by these tissues. However, rabbit bone marrow is able to take up chylomicron REs in the absence of LPL activity [245].

2.6.4.2 Uptake of REs in CRs by Liver Hepatocytes

The liver is the major organ in the body for the storage and metabolism of VA. In fact, more than 90% of the body's VA is found in the liver. Two types of

liver cells are involved in hepatic VA metabolism: hepatocytes (parenchymal cells) and stellate cells.

Hepatocytes are thought to contain at least two plasma membrane receptors that can bind CRs with high affinity: B/E receptor that binds lipoproteins containing either apolipoprotein (apo) B or apoE, and apoE receptor that specifically binds apoE containing lipoproteins. It was reported that CRs were preferentially cleared by receptors that have high affinity for the apoE moiety [246]. ApoE deficient mice display very high circulating levels of total cholesteryl and REs even in the fasting state, indicating the critical role of apoE receptor in the uptake of CRs [247]. Several distinct cell surface receptors that are able to bind apoE-containing lipoproteins may be involved in the uptake of CRs by hepatocytes. Other than low-density lipoprotein receptor (LDLR, or B/E receptor), the LDLR-related protein (LRP), lipolysis-stimulated receptor (LSR), lipoprotein lipase and hepatic lipase may also play a role [248, 249].

2.6.4.3 Fate of REs in CRs in Hepatocytes

Lipoproteins are taken up into the hepatocytes via receptor-mediated endocytosis. The receptors are normally associated with defined locations on the cell surface. For example, LDLRs are normally present in clathrin-coated pits on the cell surface, which when bound to CRs via adaptin, are pinched off to form clathrin-coated vesicles inside the cell [250]. Soon after the internalization of the CRs-receptor complex, the newly endocytosed retinyl esters are hydrolyzed to retinol by bile salt-independent hydrolase at the plasma membrane and by acid hydrolase in the early endosomes [251]. This process is rapid so that the REs will not be delivery to lysosomes that other ligands are transferred to. The receptors are then either destroyed or they can be recycled via the endocytic cycle back to the surface of the cell where the neutral pH will cause the receptors to revert to its native conformation [250]. In contrast, newly formed retinol is bound to CRBP-I and the complex is delivered to endoplasmic reticulum, where retinol binding protein (RBP) is

found in high concentration [252]. Binding of retinol-CRBP-I to RBP initiates a translocation of the holo-RBP to Golgi, followed by secretion from the cells [253].

2.6.4.4 Transfer of Retinol from Hepatocytes to Stellate Cells

It was reported that when chylomicrons labeled with [^{3}H]retinyl palmitate or with retinyl [^{3}H]palmitate were injected intravenously into rats, both retinol and palmitate moiety were detected in hepatocytes. However, only the retinol moiety was detected in stellate cells, indicating that the REs are hydrolyzed before retinol is transferred to stellate cells. In addition, antibodies against RBP completely block the transfer of retinol from hepatocytes to stellate cells, suggesting that RBP is critical to the transfer [254, 255]. Under normal conditions, most of retinol is found in stellate cells, the major cellular site of VA storage in the body. It has been estimated that approximately 5-30% of the total retinol in the livers of rats is present in hepatocytes, with the reminder in stellate cells [256]. However, as hepatic VA store decline, the proportion of VA in hepatocytes increases relative to hepatic stellate cells. It has been shown that in VA deficient (VAD) rats, little chylomicron remnant-derived retinol was found in stellate cells, possibly due to the little transfer from hepatocytes or to the rapid release from stellate, indicating that the transfer of retinol to stellate cell is influenced by VA status [257]. A minor fraction of the newly absorbed retinol that is rapidly secreted from hepatocytes escapes the uptake mechanism of stellate cells and reaches the blood [225].

2.6.4.5 Metabolism of VA in Stellate Cells

After entering into the stellate cells, retinol is re-esterified into REs. As in the intestine, both LRAT and ARAT may be involved in retinol esterification in stellate cells, which are highly enriched in CRBP-I and LRAT. When retinol is present in normal amounts, it is bound to CRBP-I and esterified by LRAT. When retinol is present at high levels and CRBP-I becomes saturated, ARAT may esterify the excess [217].

In the vitamin A-sufficient (VAS) adult rats, 70-95% of hepatic VA is stored as REs in lipid droplets of stellate cells. Approximately 99% of the VA present in stellate cells is present as REs. It was reported that the lipid of droplets isolated from VAS rats consisted of approximately 42% RE, 28% TG, 13% total cholesterol (free and ester) and 4% phospholipid. The REs in the droplets consisted of approximately 70% retinyl palmitate, 15% retinyl stearate, 8% retinyl oleate, 4% retinyl linoleate and smaller percentages of other long-chain REs [258].

2.6.4.6 Retinol Mobilization

It has been shown that RBP is required for mobilization and storage of retinol. RBP, mainly found in the liver, belongs to the lipocalin protein family and is able to bind to and protect retinol from being metabolized, due to a special hydrophobic pocket [259]. It has been shown that RBP recycles extensively between the liver, plasma, and extrahepatic tissues [238]. The retinol levels in the blood of mice null for RBP (*rbp*-/-) are much lower than those of wild-type littermates, and in the liver there is increased storage of retinyl esters. In addition, by 5 month of age, *rbp*-/- mice accumulate much higher stores of retinol than their wild-type littermates, and their hepatic stores do not change after a short-term exposure to a VAD diet [260].

The concentration of the RBP-retinol complex in blood is regulated within the physiological range (~ 1.4 μM) by stellate cells through the controlled uptake and release of retinol [261]. REs need to be hydrolyzed before the mobilization of retinol from stellate cells to ensure constant supply of peripheral tissues with retinol. This is accomplished by RE hydrolyses. However, little is known about the enzymes and mechanisms how REs are mobilized and how these processes are regulated [262]. Early work demonstrated that retinyl palmitate hydrolase played a partial role [263, 264]. The mechanisms for retinol mobilization from stellate cells have been controversial. First, retinol is bound to endogenously synthesized or internalized RBP, then secreted associated with this carrier protein in blood

circulation, without direct involvement of the hepatocytes [265]. Second, retinol is transferred from stellate cells to hepatocytes before secretion of retinol-RBP from the hepatocytes [266]. In the third hypothesis, retinol mobilization from stellate cells does not involve RBP synthesis and secretion [267].

2.6.4.7 Transport of Retinol in Plasma

Retinol in plasma is bound to a specific plasma transport protein, RBP, for the transport to peripheral tissues [268]. The retinol-RBP complex is bound to another plasma protein, transthyretin (TTR). It is believed that formation of retinol-RBP-TTR complex reduces the glomerular filtration of retinol and renal catabolism of RBP. In addition, retinoic acid circulates in plasma bound to albumin, and low levels of RE can be found in the plasma fractions of lipoproteins, including VLDL, LDL, and HDL.

2.6.4.8 Cellular Uptake of Retinol

The circulating retinol-RBP is taken up by peripheral tissues via a receptor that recognizes RBP. The existence of a specific receptor for RBP on the retinal pigment epithelium was suggested more than 35 years ago [269]. During the past 3 decades, evidence has accumulated for the existence of the RBP receptor on other tissue or cell types, including the placenta, choroid plexus, testis, and macrophages [270]. However, the molecular mechanism by which retinol is taken up by cells from the retinol-RBP complex was not understood until STRA6 was identified in bovine retinal pigment epithelium cells in 2007 [270]. STRA6 is a member of a large group of “stimulated by retinoic acid” genes that encode transmembrane proteins and other proteins whose functions are largely unknown [271]. They provided evidence that STRA6 acts as a high-affinity cell-surface receptor for RBP and proposed that STRA6 is a major physiological mediator of retinol uptake by cells [272].

STRA6 is widely expressed in the murine embryo and in the adult. Particularly strong expression of STRA6 occurred in cells that compose

human blood-organ barriers (*e.g.*, the brain, eye, testis, kidney, spleen, and female reproductive tract) [273]. Mutations in human STRA6 are associated with severe pathological phenotypes in many organs such as the eye, brain, heart, and lung [274]. More importantly, the phenotypes observed for STRA6 absence are more severe than those for RBP [275, 276], suggesting that STRA6 may play other roles beyond mediating retinoid signaling. In fact, it has been shown recently that association of RBP-retinol with STRA6 triggered tyrosine phosphorylation and resulted in recruitment and activation of JAK2 and the transcription factor STAT5, leading to induced expression of STAT target genes, including suppressor of cytokine signaling 3 (SOCS3) that inhibits insulin signaling and PPARγ that enhances lipid accumulation both *in vitro* and *in vivo* [277].

2.6.5 Cellular Metabolism of Retinoids

2.6.5.1 Retinol Processing

The major source of the synthesis of active retinoid metabolites in cells of vertebrates is all-*trans* retinol taken up from plasma. However, retinol derived from the circulating lipoproteins containing REs, carotenoids, and REs locally stored in lipid droplets in the target cells themselves or neighboring cells is also thought to contribute to the synthesis of active retinoid metabolites. In addition, all-*trans* retinoic acid or its metabolites can also be taken from plasma [238].

In the target cells, free retinol and CRBP-I-bound retinol is oxidized to retinal (retinaldehyde) by cytosolic medium-chain alcohol dehydrogenases (ADHs) and membrane-bound short-chain dehydrogenases (SDRs), respectively [278]. There are three ADHs: ADH1, ADH3, and ADH4. ADH3 is ubiquitously expressed, whereas the expression of ADH1 and ADH4 is tissue-restricted. It has been shown that ADH3 functions as a ubiquitous dehydrogenase, whereas ADH1 and ADH4 are necessary only in extreme conditions such as VA excess or deficiency [279]. Mice lack for ADH3 (*Adh3*$^{-/-}$) have reduced viability and growth when maintained on a standard VAS diet,

but this phenotype was rescued by placement on a retinol-supplemented diet, indicating that other retinol-oxidizing enzymes can compensate for the ADH3 function if retinol is present at high enough levels [280]. *Adh1*$^{-/-}$ and *Adh4*$^{-/-}$ mice do not exhibit defects in growth or survival when maintained on a VAS diet [281, 282].

There are two major groups of human SDR proteins based on their cofactor preference. One group of SDR comprises the enzymes with high affinities for NAD^+/NADH, including 11-*cis*-retinol dehydrogenase (RDH), RDH4, RDH-like 3α-hydroxysteroid dehydrogenase, and RDH-like SDR. The other group of SDR proteins have high affinities for $NADP^+$/NADPH, including RDH11, RDH12, RDH14, retinal SDR1, photoreceptor RDH, and RDH10 [279].

2.6.5.2 Retinal Processing

Retinal can be reversibly reduced to produce retinol or it can be irreversibly oxidized to produce RA, the latter is accomplished by retinal dehydrogenases (RALDHs) [283]. Vertebrates generally have four RALDHs: RALDH1-4. An additional enzyme called ALDH1A4 and ALDH1A7 is present in rat and in mouse, respectively [284].

RALDH1, also called ALDH1A1, is highly expressed in the dorsal retina of embryos and in several adult epithelial tissues [285]. The dorsal retina of *Raldh1*$^{-/-}$ embryos display only minor effects, suggesting that RALDH1 is not essential for RA synthesis in most tissues but may instead be involved in the catabolism of excess retinol [238, 279, 286]. Recently, it has been shown that RALDH1 and its substrate retinal are determinants of the adipocyte plasticity and adaptive thermogenesis in mice. White adipose tissue (WAT)-selective *Raldh1* knockdown can induce a brown adipose tissue-like transcriptional program in WAT that drives uncoupled respiration and adaptive thermogenesis, therefore limiting weight gain and improving glucose homeostasis, suggesting that disruption of *Raldh1* expression or function in

visceral fat could be a previously unrecognized target for treating obesity-related complications [287].

RALDH2, also called ALDH1A2, is expressed in multiple embryonic and adult tissues. *Raldh2*$^{-/-}$ mice die at mid-gestation, around day E8.75, due to defects in heart development. However, the *Raldh2*$^{-/-}$ embryos can be rescued to a considerable extent with external administration of RA [288], suggesting a critical role of RALDH2 in RA synthesis during embryonic development.

RALDH3, also called ALDH1A3, is expressed in mouse and chicken retina, lens and olfactory pit, as well as ureteric buds and surface ectoderm over the developing forebrain [279]. It has been shown that RALDH3 alone may supply sufficient RA to control eye development in *Raldh1*$^{-/-}$ mice, and contribute to the rescue of *Raldh2*$^{-/-}$ mice embryos [279].

RALDH4 is expressed in mouse liver and kidney and displays a preference for 9-*cis* retinal over all-*trans* retinal [289]. Thus, it was suggested that RALDH4 is involved in the synthesis of 9-*cis* RA [238].

Although both ALDH1A4 and ALDH1A7 have high sequence similarity with that of RALDH1, neither of them functions in RA synthesis [290, 291].

Newly synthesized RA is bound to cellular RA binding proteins types I and II (CRABP-I and CRABP-II) and can then either enter the nucleus to activate transcription or be transported to a nearby target cell [286]. In the mouse embryo, both isoforms of CRABP are widely expressed; in the adult, CRABP-I is expressed almost ubiquitously, whereas CRABP-II is only expressed in skin, uterus, ovary, and in the choroid plexus [292]. It was suggested that CRABPs serve to solubilize and protect RA in the cytosol, and deliver it to the nucleus [293]. However, mice deficient in CRABP-I, in CRABP-II, or in both CRABP-I and CRABP-II are essentially normal [294, 295], suggesting a possible compensatory effect by other cellular proteins that can bind to RA.

2.6.5.3 RA Metabolism

Due to the potent activity of RA in the expression of genes involved in a variety of physiological processes, its level should be delicately regulated. It is believed that the catabolism of RA is critical to the maintenance of RA in cells and tissues. In vertebrates, three cytochrome P450 enzymes (CYP26s), designated CYP26A1, CYP26B1, and CYP26C1, are able to metabolize RA to polar metabolites, such as retinoyl β-glucuronide, 5,6-epoxyretinoic acid, 4-hydroxyretinoic acid, 4-oxoretinoic acid, and 3,4-didehydroretinoic acid [296].

CYP26A1 is highly expressed in the liver, duodenum, colon, and placenta and in some regions of the brain [238]. The *Cyp26a1* null mice die during mid- to late-gestation and display a number of morphogenetic defects, which resemble those observed in RA teratogenicity [297]. It has been shown that *Cyp26al* is a RA responsive gene and that VAD rats exhibit significantly lower mRNA expression of *Cyp26al* compared to pair-fed VAS rats [298].

CYP26B1 has a different tissue expression pattern than CYP26A1, even though the two enzymes have similar catalytic activity [299]. The *Cyp26b1* null mice display severe limb malformations [300].

CYP26C1 metabolizes all-*trans* RA to polar metabolites similar to those generated by CYP26A1 and CYP26B1. In the developing mouse embryo, CYP26C1 functions to protect the hindbrain, first branchial arch, developing ear, and tooth buds from RA exposure [300]. However, CYP26C1 can catabolize 9-*cis* RA much better than CYP26A1 and CYP26B1 [238].

The expression patterns of the three CYP26s are non-overlapping, suggesting individual roles for each of the enzymes in the catabolism of RA in different tissues [301]. In addition, the mRNA expression of *Cyp26a1* and *Cyp26c1* can be induced in the presence of RA, indicating a mechanism through which the CYP26s sense the concentration of RA and regulate the catabolism of RA accordingly [238].

2.6.6 Retinoids Signaling

It is generally accepted that most of the activities of VA are mediated by its metabolites all-*trans* and 9-*cis* RA via the regulation of gene expression. This is achieved by the binding of RA to two classes of the superfamily of NRs, RARs and RXRs. NRs represent a family of transcription factors that mediate a complex array of extracellular signals into transcriptional responses in a ligand-dependent manner. Other than VA, this family includes receptors for endocrine steroids, vitamin D, thyroid hormone, and a large number of "orphan" receptors, whose ligands, target genes, and physiological functions were initially unknown [162, 175-177]. Upon ligands binding, nuclear receptors undergo a conformational change that coordinately dissociates co-repressors and facilitates recruitment of co-activators to enable transcriptional activation [162]. Three types of RARs (RARα, β, and γ) bind and respond to all-*trans* and 9-*cis* RA, and RXRs (RXRα, β, γ) can only bind and respond to 9-*cis* RA. RAR/RXR heterodimers and RXR/RXR homodimers modulate gene expression by binding to RA responsive elements (RAREs) located in the regulatory regions of target genes [302]. Most of the naturally occurring RAREs consist of the direct repeats of two core motif (A/G)G(G/T)TCA separated by 5 (DR5), 2(DR2) or 1 bp (DR1) [178]. When bound to DR2 and DR5 elements, the 5' half-site is occupied by RXR and the 3' half-site by RAR. In contrast, when bound to DR1 elements, the polarity of the heterodimer is inverted and the complex is unresponsive to RA stimulation, probably due to the inability of RAR ligands to induce the dissociation of co-repressors [286]. It is believed that the most important ligand for the RAR/RXR heterodimer is all-*trans* RA binding to the RAR heterodimer partner, since the physiological role of 9-*cis* RA has been questioned [238] (see also Sections 2.5.1 and 2.5.2).

It is noteworthy that retinoid signaling can be mediated by receptors other than the RAR/RXR heterodimer. It was recently demonstrated that RA is a potent endogenous ligand for PPARβ/δ, which functions similarly to RAR and RXR to regulate gene expression in response to retinoid [303]. In addition, analyses of the crystal structure of RA receptor-related orphan receptors

(RORβ) and chicken ovalbumin upstream promoter-transcription factor II (COUP-TFII) indicate that RA could be a potential ligand [200, 304] (see also Section 2.5.4)

2.6.7 VA in Nutrient Metabolism

Except for being crucial to vision, growth, embryological development, cell differentiation, reproduction, maintenance of mucous secretion and apoptosis, VA also plays essential role in nutrient metabolism. The status and metabolism of VA exert profound influence on the metabolism of liver, adipocytes, pancreatic β-cells, and skeletal muscle (for a review, see [10]).

2.6.8 RBP and Insulin Resistance

Liver, skeletal muscle and adipose tissue are the three major targets for the metabolic actions of insulin. Insulin regulates glucose homeostasis by reducing hepatic glucose output and by increasing the rate of glucose uptake by skeletal muscle and adipose tissue. Stimulation of glucose uptake into muscle cells and adipocytes by insulin depends largely on translocation of the glucose transporter GLUT4 from an intracellular compartment to the cell surface [305]. Recently, Yang *et al.* identified RBP4, a plasma transport protein for retinol, as a new adipokine that links glucose uptake in adipocytes with systemic insulin sensitivity [306]. Using DNA array analyses on epididymal adipose tissue RNA from two lines of mice in which GLUT4 is increased or decreased only in adipocytes, they identified *Rbp4* as one of the genes that were reciprocally regulated by the genetic manipulations and that encoded secreted proteins. They found that expression of RBP4 was increased in adipose tissue of mice with adipocyte-specific deletion of GLUT4. They also showed that circulating RBP4 levels were markedly increased not only in several mouse models of obesity and insulin resistance, but also in humans with these conditions. In addition, an insulin-sensitizing drug reduced the elevated levels of RBP4 in both adipose tissue and serum of mice. Over-expression of RBP4 or injection of recombinant RBP4 in normal mice induced

insulin resistance, whereas mice heterozygous or homozygous for knockout of the gene encoding RBP4 showed increased insulin sensitivity compared with wild-type mice. Moreover, the synthetic retinoid fenretinide, which reduced the serum levels of RBP4 through urinary excretion, ameliorated insulin resistance in mice fed a high-fat diet. These results suggest RBP4 as an adipocyte-derived signal the may contribute to the pathogenesis of T2D. Furthermore, serum RBP4 levels correlated with the magnitude of insulin resistance in subjects with obesity, impaired glucose tolerance, or T2D and in non-obese, non-diabetic subjects with a strong family history of T2D. A therapeutic intervention that improves insulin sensitivity is associated with a reduction in serum RBP4 levels [305]. Regarding the mechanism, RBP4 affected insulin action by decreasing the activity of PI3K and the phosphorylation of IRS1. Moreover, Berry *et al.* have shown that association of RBP-retinol with STRA6 can trigger tyrosine phosphorylation, resulting in recruitment and activation of JAK2 and the transcription factor STAT5, which in turn leads to inhibition of insulin signaling [277]. Although it has been proposed that the correlation between RBP4 and the magnitude of insulin resistance should be assessed in more diverse groups [307], all abovementioned findings suggest the possibility that alterations of retinol metabolism might influence the action of insulin and the risk of T2D. However, there are no compelling data to suggest that dietary VA contributes to the elevation in serum RBP4 levels observed in insulin-resistant states or to insulin resistance [305]. Moreover, a recent study showed that lowering serum RBP levels by a non-retinoid ligand for RBP did not improve insulin sensitivity in mice, and that mice null for RBP displayed normal insulin sensitivity and were not protected from high fat diet-induced insulin resistance [308].

CHAPTER III

EFFECTS OF RETINOIDS ON THE EXPRESSION OF HEPATIC *SREBP-1C*

Disclosure: The work described in this chapter in its entirety has been published in the following reference with minor modifications in the numbering of figures: Li R, Chen W, Li Y, Zhang Y, Chen G: Retinoids synergized with insulin to induce Srebp-1c expression and activated its promoter via the two liver X receptor binding sites that mediate insulin action. Biochem Biophys Res Commun 2011, 406:268-272.

3.1 Introduction

Elevation of hepatic vitamin A (VA, retinol) content in patients with diabetes was observed more than 70 years ago (1937) [309]. Subsequently, depletion of hepatic glycogen content in VA deficient (VAD) rats was reported in 1957 [310]. When isotretinoin, 13-*cis* retinoic acid, was used to treat patients with acne, some of them developed isotretinoin-induced hypertriglyceridemia [311]. All these early observations suggested that VA status affected glucose and lipid homeostasis, a topic remained to be investigated.

As an essential micronutrient, VA plays crucial roles in the general health of an individual. Therefore, retinol homeostasis must be delicately maintained to meet optimal physiological functions. This is achieved by a network of enzymes and proteins involved in the transport, production, and catabolism of retinoids [312]. The regulation of this system can be attributed to the control of the expression levels of some of these enzymes by the active metabolite of retinol, retinoic acid (RA) [313]. RA exists in multiple isomeric forms, such as all-*trans* RA and 9-*cis* RA, and RA regulates gene expression through activation of two families of nuclear receptors, retinoic acid receptors (RARα, β, and γ) activated by all-trans RA, and retinoid X receptors (RXRα, β, and γ) activated only by 9-*cis* RA [261].

Insulin resistance, diabetes and other metabolic abnormalities are associated with profound changes of hepatic lipid and glucose metabolism. These can be attributed to the altered expression of genes involved in glucose and lipid metabolism [314]. Insulin responsive elements in the *Srebp-1c*

promoter have been identified as two liver X receptor (LXR) binding sites and one sterol regulatory element [168, 315]. This implies that insulin regulates the expression of its responsive genes after it stimulates the synthesis of endogenous agonists for nuclear receptor activation. When we analyzed the effects of the lipophilic extract (LE) from rat livers on insulin-regulated gene expression, we found that the LE synergized with insulin to induce glucokinase gene (*Gck*) and *Srebp-1c* expression in primary rat hepatocytes with different induction patterns [11]. The existence of retinol and retinal in LE was confirmed later, and their effects on *Gck*, but not *Srebp-1c*, were examined in that study [11]. It has been reported that SREBP-1c mediated the retinoid-dependent increase in fatty acid synthase (*Fas*) promoter activity in HepG2 [316]. Therefore, we hypothesized that retinoids may regulate the expression of *Srebp-1c* in primary hepatocytes.

In this study, we report that retinoids transiently synergized with insulin to induce the expression of *Srebp-1c* in primary rat hepatocytes via the activation of RXR, but not RAR. The retinoic acid responsive elements (RAREs) in its promoter are the previously identified two LXR responsive elements that mediated insulin-induced *Srebp-1c* transcription.

3.2 Materials and Methods

Reagents

The reagents for primary hepatocytes isolation and culture have been published [317]. Reagents for cDNA synthesis and real time PCR were obtained from Applied Biosystems (Foster City, CA). Source of LG268 was reported previously [11]. All other compounds or enzymes were purchased from Sigma (St. Louis, MO) unless described otherwise.

Animals and Diets

Sprague-Dawley rats (for hepatocytes) were purchased from Harlan Breeders (Indianapolis, IN). Rats were housed in colony cages, and fed a standard rodent diet before isolation of primary hepatocytes. All procedures

were approved by the Institutional Animal Care and Use Committee at the University of Tennessee at Knoxville.

Primary Hepatocytes, RNA Extraction and Quantitative Real-time PCR

Methods for preparation of primary hepatocytes and analysis of RNA were described previously [11]. The real time PCR primer sets for detecting *Fas* (from Dr. Bruce Spigelman's group in Harvard Medical School), *Gck*, *Cyp26a1* [298], *Srebp-1c* [11] have been published. The primers for *Rarb* (forward 5'-GGCCTCTGGGACAAATTCAG-3', and reverse 5'-GCAGACGCTTGGCGAACT-3) were designed using Primer Express software (Applied Biosystems). The gene expression level was normalized to that of 36B4 unless described otherwise. Data were presented as the fold induction calculated from the ΔΔCt values [317] using 36B4 as the invariable control gene [11].

INS-1 Cell Culture, Reporter Gene Constructs and Assay

INS-1 cells (833/15) were maintained as described previously [318]. Standard protocols (Molecular Cloning) were followed in all recombinant DNA engineering procedures. The reporter gene constructs reported previously [168] were transfected into INS-1 cells using Fugene 6 transfection reagent (Roche, Indianapolis, IN) according to the manufacturer's manual. The activation of reporter gene constructs were measured using dual luciferase assay as described previously [168] and reported as fold induction.

Statistics

Data were presented as means ± SD. The number of experiments represented the independent experiments using hepatocytes isolated from different animals on different days. Levene's test was used to determine homogeneity of variance among groups using SPSS 17.0 statistical software and where necessary natural log transformation was performed before analysis. Multiple comparisons were analyzed by one-way ANOVA. The

independent sample t-test was used to compare two conditions. Differences were considered statistically significant at $P < 0.05$.

3.3 Results

Retinal and RA Synergized with Insulin to Induce *Srebp-1c* Expression

Since we have observed that rat liver LE that contained retinol (ROL) and retinal (RAL) synergized with insulin to induce *Gck* and *Srebp-1c* expression with different induction patterns, we only reported the effects of ROL, RAL, and RA on *Gck* expression in the previous publication [11]. We decided to check the direct effects of retinoids on the expression level of *Srebp-1c*, a key transcription factor controlling the hepatic fatty acid biosynthesis [131]. Primary rat hepatocytes were treated with increasing concentrations of ROL, RAL, and RA in the absence or presence of insulin. As shown in Fig. 3.1, retinol up to 20 μM did not induce *Srebp-1c* expression without or with insulin. In the absence of insulin, RAL up to 20 μM did not affect *Srebp-1c* expression. RAL synergized with insulin to induce *Srebp-1c* expression when its concentration reached 20 μM. Without insulin, RA at 20 μM induced *Srebp-1c* expression. RA at 2 and 20 μM synergized with insulin to induce *Srebp-1c* expression. All these results demonstrated that RAL and RA had the ability to synergize with insulin to induce *Srebp-1c* expression in primary rat hepatocytes.

RA Transiently Synergized with Insulin to Induce the Expression of *Gck* and *Srebp-1c* Differently, and Resulted in Elevation of SREBP-1c Target Gene, *Fas*

Since RA induced the expression of both *Gck* and *Srebp-1c*, it is important to determine whether their induction patterns are similar or not. The expression levels of *Gck*, *Srebp-1c*, and *Fas*, a target gene of SREBP-1c, were examined by real time PCR at 0, 3, 9, 12, and 24 h after treatment of 5 μM RA in the absence or presence of 1 nM insulin. As shown in Fig. 3.2A, RA robustly synergized with insulin to induce *Gck* expression as early as three

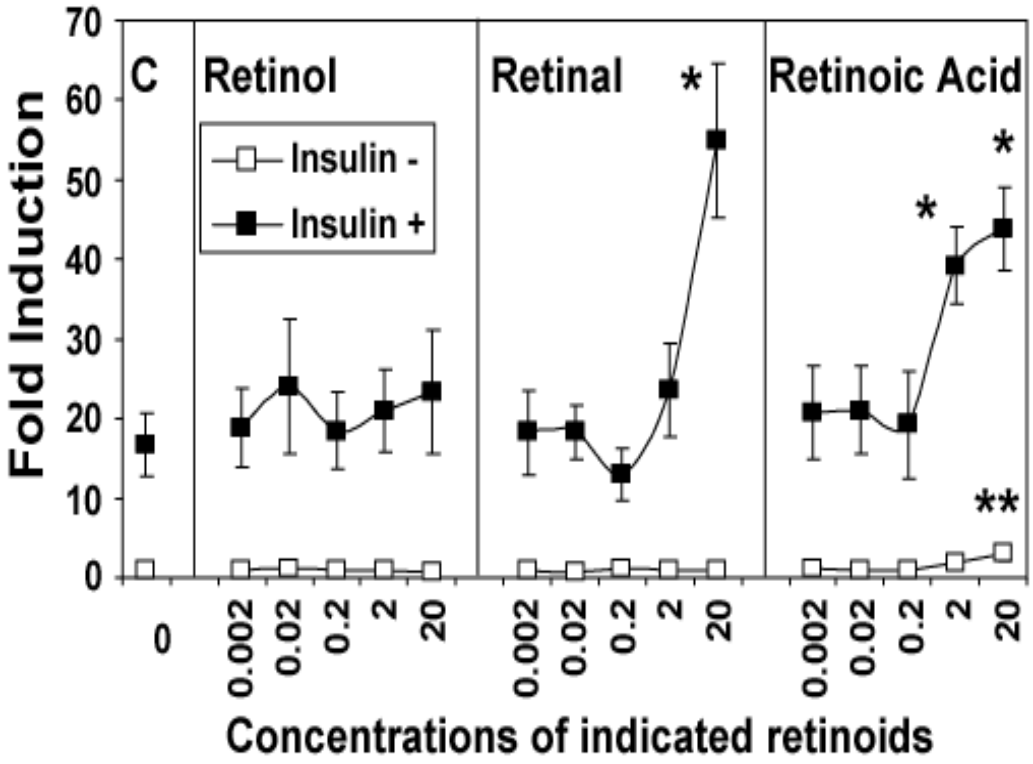

Figure 3.1 Retinal and RA synergized with insulin to induce *Srebp-1c* mRNA expression in rat primary hepatocytes

Hepatocytes were treated with indicated ligands (μM) without or with 1 nM insulin for 6 h. Total RNA was isolated and subjected to real-time PCR analysis. *Srebp-1c* mRNA level in vehicle control group was assigned a value of 1 (mean ± SD, n = 3, * for comparing indicated groups with control in the presence of insulin; ** for comparing RA group with control group in the absence of insulin; all $P < 0.05$).

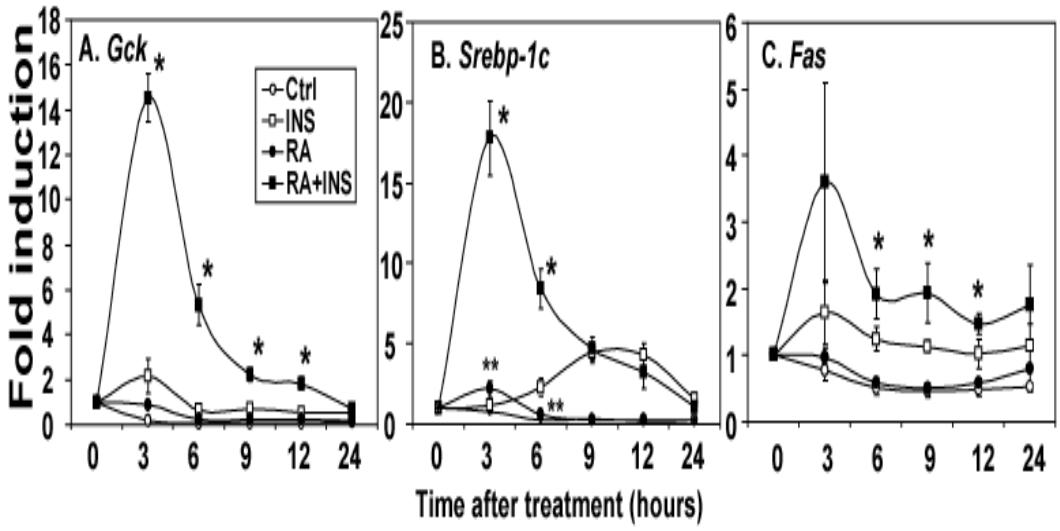

Figure 3.2 Comparison of the effects of RA (5 μM) and insulin (1 nM) on the expression levels of *Gck* (A), *Srebp-1c* (B), and *Fas* (C) mRNA over time

Hepatocytes were treated with or without RA in the absence or presence of insulin. The expression level of indicated transcripts at time 0 was assigned a value of 1 (mean ± SD, n = 3, * for comparing RA + insulin with insulin treatment of indicated gene at the indicated time points; ** for comparing RA with control group of *Srebp-1c* at the indicated time points; all $P < 0.05$).

hours. The fold induction started to decline at 6 h after the stimulation, and the synergy lasted for at least 12 h. Fig. 3.2B showed that hepatocytes treated with 5 μM RA had significantly higher levels of *Srebp-1c* mRNA than control cells did at 3 (2.1 ± 0.5- vs 0.71 ± 0.07-fold) and 6 (0.53 ± 0.09- vs 0.26 ± 0.02-fold) hours after treatment. RA also robustly synergized with insulin to induce *Srebp-1c* expression at 3 h and the fold induction began to drop at 6 h, similar to the pattern of *Gck* expression. However, at 9 h after the stimulation, the synergistic induction of RA and insulin to *Srebp-1c* expression no longer existed. As shown in Fig.3.2C, RA + insulin significantly induced *Fas* expression to a higher level than insulin alone did at 6, 9 and 12 h, suggesting that the rapid and robust increase of *Srebp-1c* downstream gene. All these results demonstrated that RA, the active metabolite of VA, synergized with insulin to induce glycolytic and lipogenic genes differentially, probably with different induction mechanisms.

Activation of RXR, but not RAR, Synergized with Insulin to Induce *Srebp-1c* Expression in Primary Hepatocytes

RA regulates gene expression through activation of RARs and RXRs [261]. To determine whether one or both of these nuclear receptors mediate the synergistic effect of RA and insulin on *Srebp-1c* expression, primary hepatocytes were treated with TTNPB (1 μM), a specific agonist of RARs, LG 268 (1 μM), an RXR specific agonist, or the combination of both. Fig. 3.3A showed that TTNPB induced the expression of *Cyp26a1*, a RA responsive gene [298], by 6500 ± 1300- and 6200 ± 1600-fold in the absence and presence of insulin (1 nM), respectively. Activation of RXR by LG268 only induced *Cyp26a1* moderately in the absence (15 ± 5.3-fold) and presence (12 ± 4.2-fold) of insulin. In comparison to TTNPB alone, the combination of TTNPB and LG268 further induced the *Cyp26a1* expression in the absence (27,000 ± 12,000-fold) and presence (28,000 ± 8700-fold) of insulin. The enormous induction of *Cyp26a1* expression by TTNPB showed the extreme

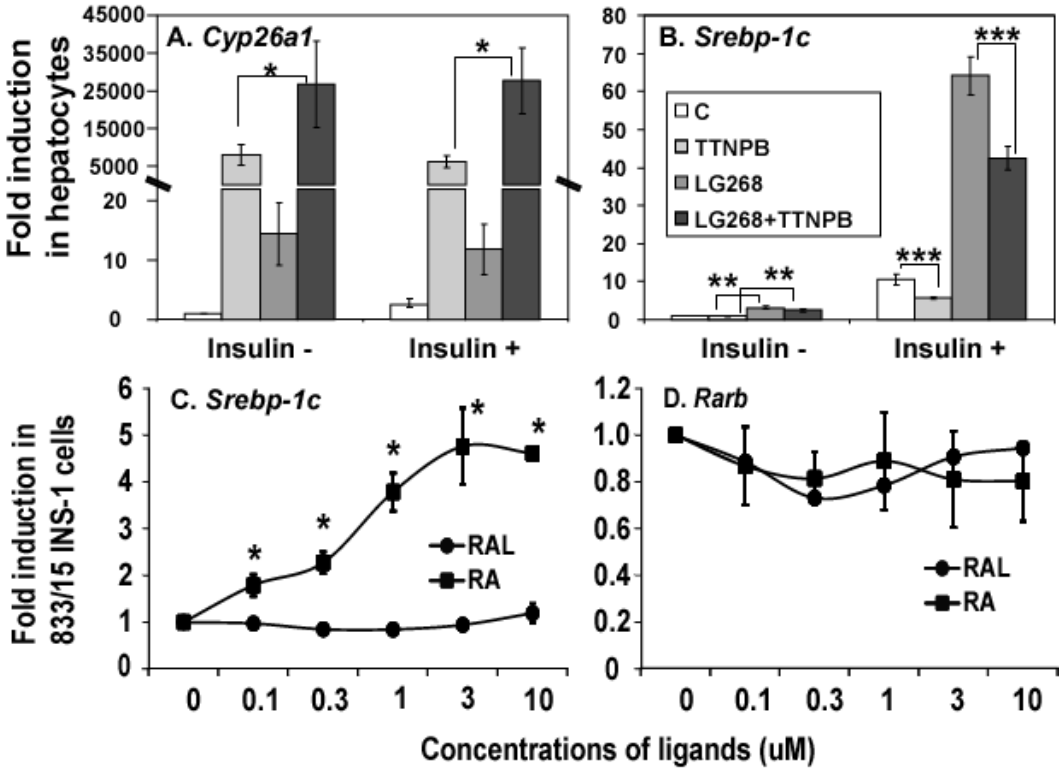

Figure 3.3 The expression levels of *Cyp16a1* (A) and *Srebp-1c* (B) in primary hepatocytes treated with RAR and RXR agonists, and *Srebp-1c* (C) and *Rarb* (D) in INS-1 cells treated with RA

(A and B) Primary hepatocytes were treated with vehicle, TTNPB (1 μM), LG268 (1 μM), and TTNPB + LG268 in the absence and presence of insulin (1 nM) for 6 h. The expression level of the vehicle control group was arbitrarily assigned a value of 1 (mean ± SD, n = 3, * $P < 0.05$ for comparing TTNPB group with TTNPB ±LG268 group of *Cyp26a1* in the absence or presence of insulin; ** $P < 0.03$ for comparing TTNPB with LG268 or LG268 + TTNPB group of *Srebp-1c* in the absence of insulin; *** $P < 0.04$ for comparing control with TTNPB or LG268 with LG268 + TTNPB in the presence of insulin). (C and D) 833/15 INS-1 cells were treated with increasing concentrations of RA or RAL for 6 h. The expression level of the vehicle control group was arbitrarily assigned a value of 1 (mean ± SD, n = 3, $^{\#}P < 0.05$ for comparing RA and RAL groups at indicated concentrations).

sensitivity of this gene to the activation of RAR and the efficacy of this compound.

Fig.3.3B showed that LG 268 (3.2 ± 0.3-fold) and LG268 + TTNPB (2.5 ± 0.5-fold), but not TTNPB, induced *Srebp-1c* expression in the absence of insulin (10.5 ± 1.5- to 5.7 ± 0.3-fold). However, LG268 synergized with insulin to induce *Srebp-1c* expression. The synergy was attenuated in the presence of TTNPB (from 64 ± 4.9- to 42 ± 3.1-fold). These results demonstrated that activation of RXR potentiated insulin-mediated induction of *Srebp-1c* expression. It suggests that RA synergizes with insulin to induce *Srebp-1c* expression through activation of RXR in primary rat hepatocytes.

RA, but not RAL, Induced *Srebp-1c*, but not *Rarb*, in a Dose Dependent Manner in 833/15 INS-1 Cells

To test the effects of retinoids on *Srebp-1c* expression in other cells, we treated 833/15 INS-1 cells with increasing concentrations of RAL and RA. Fig.3.3C showed that RA started to significantly induce *Srebp-1c* expression in INS-1 cells at 0.1 μM, the lowest concentration tested. On the other hand, RAL did not change *Srebp-1c* expression level at any concentration tested. Due to the extremely low expression level of *Cyp26a1* in 833/15 INS-1 cells, we examined the effects of RAL and RA on the expression of retinoid acid receptor beta isoform gene, *Rarb*, a RA responsive gene [319]. Fig.3.3D showed that RAL or RA could not change the expression of *Rarb* at any concentration tested, suggesting the activation of RXR, but not RAR by RA in INS-1 cells. These results demonstrated that INS-1 cells can be used as a tool for investigating RA-induced *Srebp-1c* expression.

RA Activated *Srebp-1c* Transcription via the LXREs in its Promoter

Given the fact that retinoids synergized with insulin to induce its expression in primary hepatocytes, it is critical to determine the RAREs in its promoter. We have examined primary rat hepatocytes, hepatoma cells and INS-1 insulinoma cells for their potential use in the determination of the RA-

induced activation of the *Srebp-1c* promoter reporter constructs in them. Subsequently, we found that RA robustly induced the activation of *Srebp-1c* promoter reporter constructs in INS-1 cells, and decided to use these cells for identification of RAREs in its promoter. As shown in the left panel of Fig. 3.4, a series of *Srebp-1c* promoter reporter gene constructs [168], plasmid D (wild type), m9 (LXRE1 mutant), m13 (LXRE2 mutant), m24 (SRE mutant), m31 (LXRE1/LXRE2 mutant), and m34 (LXRE1/LXRE2/SRE mutant) were made previously for identification of insulin responsive elements in it. The RA-induced activation of these constructs in INS-1 cells was measured. Fig. 3.4 showed that both RA and T1317 (a positive control for LXR activation) induced the activation of plasmid D, which contained wild type sequence of *Srebp-1c* promoter fragment, by 2.6- and 2.2-fold, respectively. The disruption of SRE as in plasmid m24 significantly improved the fold induction mediated by either RA or T1317. This improvement was due to the reduced activation of this promoter construct in basal condition as shown previously [168]. The disruption of LXRE1 (m9) did not significantly affect RA response of this construct, but significantly reduced its response to T1317. The disruption of LXRE2 (m13) significantly reduced the response of this promoter construct of RA and T1317. The disruption of LXRE1/2 (m31) or LXREs plus SRE (m34) completely abolished the response to both RA and T1317, indicating the critical role of these two previously identified LXREs in mediating RA-induced activation of *Srebp-1c* promoter.

3.4 Discussion

We initiated our investigation based on the following reasons. First, we previously reported that the rat liver LE synergized with insulin to induce *Gck* and *Srebp-1c* expression in primary hepatocytes [11]. However, the induction patterns of these two genes by LE were different, suggesting two distinct mechanisms for the induction. After identification of retinol and retinal in the LE, only the effects of retinoids of *Gck*, but not *Srebp-1c*, were investigated and reported in that paper [11]. Given the different induction patterns of these

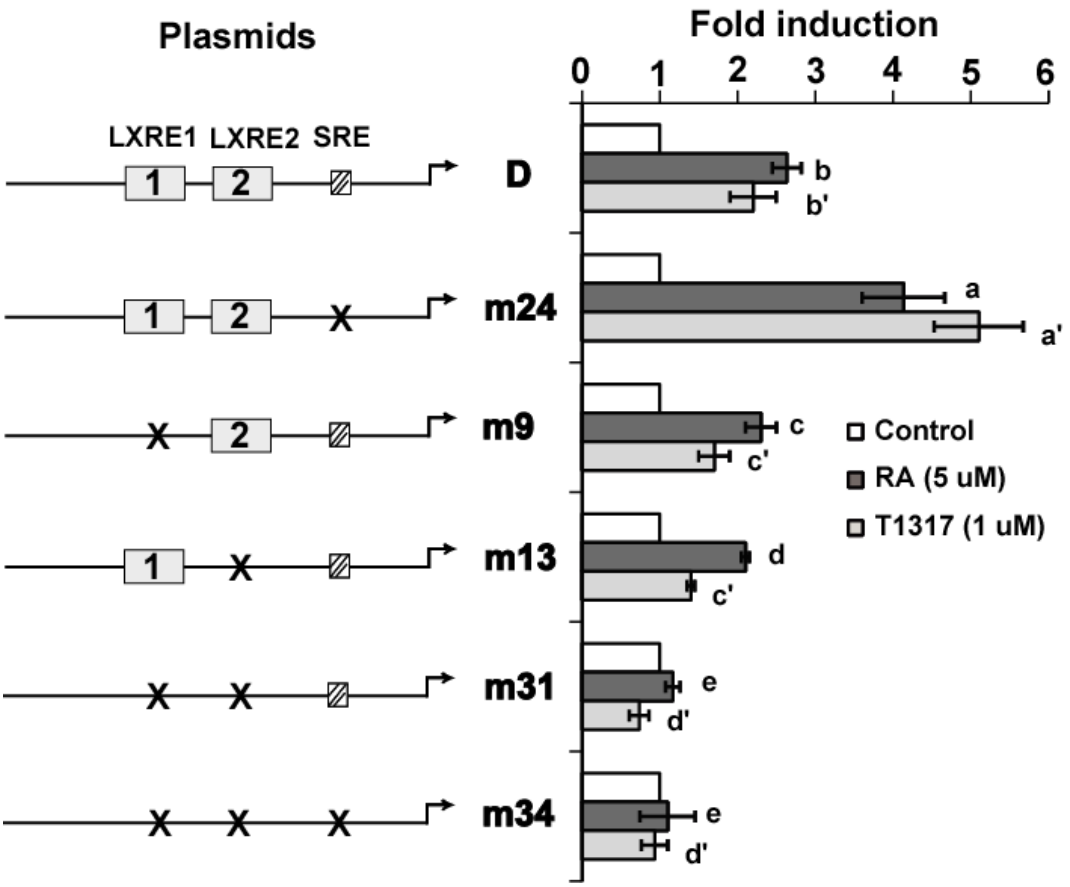

Figure 3.4 The effects of RA and T1317 on activation of Srebp-1c promoter reporter gene constructs containing indicated wild type or mutant sequences of its promoter in INS-1 cells

The indicated reporter gene constructs were transfected into INS-1 cells, and dual luciferase assay was performed to determine their activations by RA (5 μM) and T1317 (1 μM). The relative luciferase activity of each constructs treated with ethanol vehicle control was arbitrarily assigned a value of 1 (mean ± SD, n = 3, a > b > d > e, a > c > e, a' > b' > c' > d', all $P < 0.05$).

two genes mediated by LE treatments and importance of SREBP-1c in lipogenesis, it was reasonable to further investigate the effects of ROL, RAL, and RA on *Srebp-1c* expression in primary hepatocytes without or with insulin. Second, since identification of LXREs in its promoter [167, 320], recognition of endogenous ligands for LXR activation [321], and the roles of LXRE in mediating insulin-induced its expression [168, 315], *Srebp-1c* has been considered as a downstream target gene of LXR activation. On the other hands, these sites are also responsible for the effects of dietary signals, such as polyunsaturated fatty acids, on *Srebp-1c* expression in hepatocytes [168, 322]. Since RXR is heterodimer partner of LXR and can be activated by retinoids [298], it is reasonable to investigate the response of *Srebp-1c* expression to retinoids that mediate nutritional signals from vitamin A. Therefore, we reported the synergistic effects of retinoids with insulin to induce *Srebp-1c* expression, and the identification of RAREs in its promoter. In addition, we also discussed the differential induction patterns of *Gck* and *Srebp-1c* by RA in the current manuscript.

We observed that RAL and RA, but not ROL, had the capability to synergize with insulin to induce *Srebp-1c* expression (Fig. 3.1). It differed from the induction of *Gck*, which was induced by ROL, RAL, and RA in the same experimental settings [11], suggesting different induction mechanisms. Additionally, it seems that RA was more effective than RAL to induce *Srebp-1c*, implying involvement of retinoid metabolism. In INS-1 cells (Fig.3.3C), RA induced *Srebp-1c* expression at 0.1 μM, much lower than that in hepatocytes. However, RAL had no effects on *Srebp-1c* expression in them. This suggests that INS-1 cells lack the retinoid metabolism. Whether RA production plays any role in this phenomenon is under investigation.

RA-induced *Srebp-1c* expression only lasted less than 9 h in primary hepatocytes without or with insulin (Fig.3.2B). This differed from that of *Gck* expression, which lasted for more than 12 h (Fig. 3.2A). The activation of RAR and RXR may play a role here. For *Gck*, activation of both RAR and RXR was responsible for the induction [11]. For *Srebp-1c*, only activation of RXR was

responsible (Fig. 3.3B). It has been shown that mouse hepatic *Srebp-1c* expression was regulated by activation of LXR and RXR [167], which may be responsible for the regulation of hepatic *Srebp-1c* expression by dietary components, such as cholesterol [167] and polyunsaturated fatty acids [323]. Liver specific knock out of RXRα altered hepatic lipid metabolism in mice [324]. Their hepatic expression levels of RARβ and RARγ were elevated, suggesting changes of retinoid signaling as RARβ expression can be induced by RA [319]. All these imply that retinoids differentially regulate gene expression, probably through activation of different sets of nuclear receptors on their promoters. It also demonstrates that rapid and dynamic changes of transcription machinery may have happened at the *Srebp-1c* promoter similar to the spatial and temporal recruitment of co-activators of nuclear receptors as reported [325]. It seems that synergy between insulin signaling and RXR activation is shorter than that of RXR when comparing expression patterns of Gck and Srebp-1c in the same conditions. Since RXRs are the heterodimeric partners for many other nuclear receptors [326], the mechanism of this transient synergy between retinoids and insulin at *Srebp-1c* promoter deserves to be investigated.

Another interesting observation was that TTNPB treatment significantly attenuated insulin-induced *Srebp-1c* expression in the absence or presence of LG268 (Fig. 3.3B). In the absence of insulin, this attenuation did not exist. However, TTNPB synergized with insulin to increase the expression of *Gck* in the absence and presence of LG268 [11]. Since TTNPB and LG268 respectively activate RARs and RXRs, it seems that RAR activation in primary hepatocytes attenuates or potentiates insulin-regulated gene expression depends on the promoter context of the gene. This may be another reason that RA synergized with insulin for less than 12 h to induce *Srebp-1c* expression (Fig. 3.2B), but for more than 12 h to induce *Gck* expression (Fig. 3.2A). The mechanism of this attenuation deserves further investigation.

It has been shown that VAD rats had lower plasma triglyceride (TG) level, and hepatic lipogenic activity than VAS rats did [327]. These may be caused

by reduction of hepatic lipogenic gene expression in VAD rats as SREBP-1c is critical for fatty acid biosynthesis [131]. Here, we identified two RAREs in *Srebp-1c* promoter (Fig. 3.4), which are also two previously identified LXREs [320] that are also part of the insulin responsive elements [168, 315]. The role of these two LXREs may be not only for mediating insulin response, but also for sensing VA status. How nutritional (retinoids) and hormonal (insulin) signals converge at the same sites in *Srebp-1c* promoter and determine the hepatic lipogenesis is worth to be investigated. This regulation is particularly important for a heterogenous population as the nutritional status probably plays an important role in the development of metabolic diseases, such as obesity and diabetes. Understanding the regulatory mechanisms in detail will provide us with more tools for the intervention of metabolic diseases.

CHAPTER IV

REGULATION OF HEPATIC *GCK* EXPRESSION BY RETINOIC ACID AND NUCLEAR RECEPTORS

4.1 Introduction

Glucose must first be phosphorylated before being utilized by cells. This reaction is catalyzed by a family of enzymes called hexokinases, which are found in different organisms ranging from bacteria to humans [328]. Mammalian hexokinase IV (D), also known as glucokinase (GK) (ATP: D-hexose 6-phosphotransferase; EC 2.7.1.1), plays a key role in maintaining glucose homoeostasis [94]. GK mutations have been associated with maturity onset diabetes of the young [329]. Whole-body or tissue-specific deletion of *Gck* in rodents demonstrated that either pancreatic β-cell or hepatic GK activity is essential for glucose homeostasis [330, 331].

As an essential micronutrient, VA (vitamin A; retinol) plays crucial roles in the general health of an individual. Therefore retinol homeostasis must be delicately maintained to meet optimal physiological functions. This homeostasis is achieved by a network of enzymes and proteins involved in the transport, production, and catabolism of retinoids [312]. The regulation of this system can be attributed to the control of the expression of some of these enzymes by the active metabolite of retinol, RA (retinoic acid) [313]. RA exists in multiple isomeric forms, such as all-*trans* RA and 9-*cis* RA, and RA regulates gene expression through activation of two families of nuclear receptors, RARs (RA receptors; RARα, β, and γ) activated by all-*trans* RA and RXRs (retinoid X receptors; RXRα, β, and γ) activated only by 9-*cis* RA [261].

Long-term regulation of hepatic GK activity is controlled by its mRNA level. Transcription of *Gck* is regulated differentially by an upstream promoter in pancreatic β-cells and a downstream promoter in hepatocytes [81, 91]. Activation of either one of them leads to the generation of a *Gck* mRNA with distinct 5' sequences derived from the tissue-specific first exon. In rat liver, *Gck* mRNA is induced by insulin and suppressed by glucagon, a counter regulatory hormone to the actions of insulin [332, 333]. Previously, we have reported that retinoids can synthesize with insulin to induce *Gck* expression in rat hepatocytes [11]. However, the underlying mechanism has not been

defined. Here, we identified a RA responsive element (RARE) in the promoter of *Gck* and investigated the regulation of *Gck* transcription by nuclear receptors (NRs) RARα, RXRα, HNF4α, and COUP-TFII.

4.2 Materials and Methods

Reagents

All compounds or enzymes were purchased from Sigma (St. Louis, MO) unless described otherwise. Liver perfusion medium (#17701038) and liver digestive buffer (#17703034) were purchased from Life Technologies (Carlsbad, CA). Dulbecco's Modification of Eagle Medium (DMEM) and RPMI1640 were purchased from Cellgro (Manassas, VA). All primary and secondary antibodies used in Western blotting were purchase from Cell Signaling (Danvers, MA). For chromatin immunoprecipitation (ChIP) and electrophoretic mobility shift assay (EMSA), antibodies against RAR (sc-773X), RXR (sc-774X), and HNF4α (sc-8987X), and IgG control (sc-2027) were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Antibody against COUP-TFII (PP-H7147-00) was obtained from Perseus Proteomics (Tokyo, Japan).

Animals

Male Zucker lean (ZL) rats were bred in the Department of Nutrition at the University of Tennessee at Knoxville (UTK, breeding protocol number 1256). Male Sprague-Dawley (SD) rats were purchased from Harlan Breeders (Indianapolis, IN) or bred at UTK (breeding protocol number 2123). Animal facility was temperature controlled and kept on 12 h light/dark cycles (light from 7 a.m. to 7 p.m.). Rats was fed a standard chow (Rodent diet # 8640, Harlan Teklad, Madison, WI) and allowed to have free access to water.

Prior to preparation of hepatocytes, rats (200-260 grams) were euthanized with carbon dioxide. All procedures were approved by the Institutional Animal Care and Use Committee at UTK (Protocol numbers: 1642

and 1582). The animals were cared for in accordance with Guide to the Care and Use of Experimental Animals.

Plasmid Constructs

Standard molecular cloning procedures were followed during the process of making all the plasmid constructs. All constructs were verified by the restriction endonuclease digestion analysis and DNA sequencing.

The -944/-4, -843/-4, 641/-4, -455/-4, and -237/-4 (relative to the translation initiation site +1) regions of the rat liver *Gck* promoter (downstream) were amplified by PCR from ZL genomic DNA using the same reverse primer oGC-738 and different forward primer oGC-739, oGC-740, oGC-742, oGC-744, and oGC-746, respectively. The generated fragments were each gel purified using the Qiaex II Gel Extraction Kit (Qiagen, Hilden, Germany), digested overnight at 37°C with *Bgl*II and *Pci*I, and inserted into pGL3 basic (Promega, Madison, WI) cut with *Bgl*II and *Nco*I to generate pGL3-*Gck*-1K (A), pGL3-*Gck*-0.8K (B), pGL3-*Gck*-0.6K (C), pGL3-*Gck*-0.4K (D), and pGL3-*Gck*-0.2K (E), respectively. Plasmid m1 was constructed by PCR, using plasmid E as a template and the primer pair oGC-816/oGC-817. The generated 251-bp fragment was gel purified, digested with *Bgl*II and *Nco*I, and inserted into the same sites of plasmid E to yield m1. Analogously, plasmid m2 was constructed by PCR using primer pair oGC-818/oGC-817. Scramble mutations in plasmids m3 to m12, m6/7-1 to m6/7-5, and m6/7-4-1 to m6/7-4-5 were generated with the QuickChange site-directed mutagenesis kit (Stratagene, La Jolla, CA). Mutagenic oligonucleotides were synthesized so that the sequences to be scrambled were located in the center of the oligonucleotide.

A ~1.4 kb rat RARα cDNA insert, which contained the complete coding regions, was excised from pCMV-Sport6ccdB (Life Technologies, Carlsbad, CA) at *KpnI* and *BamHI* sites and inserted into the pcDNA3.1(+) vector (Life Technologies) and pACCMV5 at the same sites to generate rat RARα expression vector pcDNA3.1-RARα and pACCMV5-RARα, respectively. The

rat RXRα, RXRγ, and HNF4α cDNA containing the entire coding region were amplified by PCR from SD rat primary hepatocyte cDNA, using primer pair oGC90/oGC91, oGC108/oGC109, and oGC1004/oGC1008, respectively. The amplicons were each ligated into pCR®2.1 vector using TA cloning® Kit (Life Technologies) according to the manufacture's protocol. The respective full length cDNA was excised from pCR®2.1 at *EcoRI* site and inserted into pcDNA3.1(+) or pACCMV5 at the same site to generate expression vector pcDNA3.1-RXRα, pcDNA3.1-RXRγ, pACCMV5-RXRα, pACCMV5-RXRγ, and pACCMV5-HNF4α.

See Table 4.1 for primer sequences and mutated nucleotides.

Cell Lines

HL1C rat hepatoma cells, INS-1 833/15 rat insulinoma cells, and 293 human embryonic kidney (HEK) cells were cultured as described previously [334].

HL1C cells were cultured in DMEM containing 4.5 g/L glucose, 4% (vol/vol) heat-inactivated fetal bovine serum (FBS), 100 U/ml of penicillin and 100 μg/ml streptomycin sulfate. They were incubated in 150 mm dishes with serum free DMEM containing 1 μM retinoic acid for 4 h before being collected for ChIP assays.

833/15 INS-1 cells were cultured in RPMI 1640 medium containing 2 mM L-glutamine, 1 mM sodium pyruvate, 10 mM HEPES，and 50 μM beta-mercaptoethanol, 10% (vol/vol) heat-inactivated FBS, 100 U/ml of penicillin, and 100 μg/ml streptomycin sulfate.

293 HEK cells were cultured in DMEM containing 4.5 g/L glucose, 8% (vol/vol) heat-inactivated FBS, 100 U/ml of penicillin and 100 μg/ml streptomycin sulfate.

All cell lines were cultured at 37°C and 5% CO_2.

Table 4.1 Plasmid constructs and primers with mutated nucleotides indicated by lower cases

Plasmid	Primer (oGC-)	Sequence (5'-3')
pGL3-*Gck*-1K	839	CCATGGAGATCTCCGACAGGCACCCTATGTAC
	838	GAATTCACATGTTGAGAGGACAACCCAGG
pGL3-*Gck*-0.8K	840	CCATGGAGATCTGGCCAAACCCAAAGAAAGG
	838	GAATTCACATGTTGAGAGGACAACCCAGG
pGL3-*Gck*-0.6K	842	CCATGGAGATCTGTCAGGGCAGCCAGAGGACCTG
	838	GAATTCACATGTTGAGAGGACAACCCAGG
pGL3-*Gck*-0.4K	844	CCATGGAGATCTCAGGTGAAATCCCACGAGGATC
	838	GAATTCACATGTTGAGAGGACAACCCAGG
pGL3-*Gck*-0.2K	846	CCATGGAGATCTGTGGCCTTTGTCAAACCC
	838	GAATTCACATGTTGAGAGGACAACCCAGG
m1	816	AGATCTTCCtccgatgtgcgtTCAAACCCGACC
	817	CCATGGTGAGAGGACAACCCAGG
m2	818	AGATCTTCCCTGTGGCCTTgtcacgcaatacCCCCACGTGG
	817	CCATGGTGAGAGGACAACCCAGG
m3	819	CTTTGTCAAACCCacggagcgcgctTTCTTTGTCCTGG
	820	CCAGGACAAAGAAagcgcgctccgtGGGTTTGACAAAG
m4	821	CCGACCCCACGTctgatgactggtTGGCCCTGGCCC
	822	GGGCCAGGGCCAaccagtcatcagACGTGGGGTCGGGT
m5	823	GTGGTTCTTTGTgtgccgcgaccgCCTGGCCCTGAC
	824	GTCAGGGCCAGGcggtcgcggcacACAAAGAACCACGTG
m6	825	GTCCTGGCCCTGcgtgcctgggccACCTTGTGACAC
	826	GTGTCACAAGGTggcccaggcacgCAGGGCCAGGACAAAG
m7	827	GGCCCTGGCCCcctgacatgtctACTAGGCAGGG
	828	CCCTGCCTAGTagacatgtcaggGGGCCAGGGCCAGGG

Table 4.1 Continued.

Plasmid	Primer (oGC-)	Sequence (5'-3')
m8	834	CCCTGACCTTGTGctgaggacagacGTATTTCAGGAGCCAC
	835	GCTCCTGAAATACgtctgtcctcagCACAAGGTCAGGGCC
m9	836	GTGACACTAGGCAactaggagttatAGCCACCCCTCAGGC
	837	CTGAGGGGTGGCTataactcctagtTGCCTAGTGTCACAAG
m10	838	GCAGGGTATTTCAatccggcggagcCAGGCCCGTTAGTGC
	839	CACTAACGGGCCTGgctccgccggatTGAAATACCCTGCCTAG
m11	840	CAGGAGCCACCCgcgtctgatcgcAGTGCGGAAGTCCTTG
	841	GGACTTCCGCACTgcgatcagacgcGGGTGGCTCCTGAAA
m12	842	CCCCTCAGGCCCGgcgtaagtctgaTCCTTGGCTGCCTATC
	843	GGCAGCCAAGGAtcagacttacgcCGGGCCTGAGGGGTG
m6/7-1	951	CCTGGCCCTGcgtgcGGCCCTGACCTTG
	952	GGTCAGGGCCgcacgCAGGGCCAGGAC
m6/7-2	953	GCCCTGGCCCcctggCTGACCTTGTGAC
	954	CACAAGGTCAGccaggGGGCCAGGGCCAG
m6/7-3	955	CTGGCCCTGGCggcctCCTTGTGACACTAG
	956	GTGTCACAAGGaggccGCCAGGGCCAGGG
m6/7-4	957	CCTGGCCCTGtgacaGTGACACTAGGCAT
	958	CTAGTGTCACtgtcaCAGGGCCAGGGCC
m6/7-5	959	GCCCTGACCTatgtctACTAGGCAGGG
	960	CCTGCCTAGTagacatAGGTCAGGGCCAG
m6/7-4-1	970	CCTGGCCCTGtCCTTGTGACAC
	971	GTGTCACAAGGaCAGGGCCAGG
m6/7-4-2	972	CTGGCCCTGAgCTTGTGACAC
	973	GTGTCACAAGcTCAGGGCCAG

Table 4.1 Continued.

Plasmid	Primer (oGC-)	Sequence (5'-3')
m6/7-4-3	974	CTGGCCCTGACaTTGTGACAC
	975	GTGTCACAAtGTCAGGGCCAG
m6/7-4-4	976	GGCCCTGACCcTGTGACACTAG
	977	CTAGTGTCACAgGGTCAGGGCC
m6/7-4-5	978	GCCCTGACCTaGTGACACTAG
	979	CTAGTGTCACtAGGTCAGGGC
pcDNA3.1-rRXRα	90	GGATCCCCGGAATTCATGGACACCAAACATTTCCTGCC
	91	AAGCTTGAGCTCGAGCTAGGTGGTTTGATGTGGGGCC TC
pcDNA3.1-rRXRγ	108	GCTAGCCACCATGTATGGAAATTATTCCCACTTC
	109	TCTAGATCAGGTGATCTGCAGTGGGGTC
pACCMV5-HNF4α	1004	GGATCCGTAGAGGAGAATGCGAC
	1008	GAATTCTAGATGGCTTCCTGCTTGGTG

Preparation of Primary Hepatocytes

Rat hepatocytes were isolated as described previously [334]. After euthanasia of the animal, a catheter was inserted into portal vein and connected to a peristaltic pump that is connected to liver perfusion medium and liver digestive buffer. The inferior vena cava was cut open to allow the outflow of the media at flow rate of 10 ml/min. The liver was perfused for 15 min followed by digestion for 15 min. After completion of the digestion, livers were excised from the rat and put into a tissue culture plate containing liver digest buffer for removal of connective tissues and release of hepatocytes. Medium containing hepatocytes was filtered through a cell strainer (100 μm, Fisher Scientific, Pittsburgh, PA) and the flow through was spun at 50 g for 3 minutes. The cell pellets were washed twice with 50 ml of DMEM containing 5% FBS, 100 units/ ml sodium penicillin, and 100 μg/ml strepromycin sulfate, and suspended in the same medium for assessment of cell number and viability. The isolated hepatocytes were plated onto 60- (~ 2 million cells) and 150-mm (~ 2×10^7 cells) dishes coated with rat-tail collagen type I (Life Technologies) at ~ 0.1 mg/cm^2), and incubated in 4 and 20 ml of the same medium at 37°C and 5% CO_2, respectively. After incubation for 3-4 h, the attached cells were washed once with 4 ml (for 60-mm plates) and 20 ml (for 100-mm plates) of PBS, and incubated in medium A (medium 199 with 100 nM dexamethasone, 100 nM 3,3',5-triiodo-L-thyronine (T3), 100 units/ml penicillin, and 100 μg/ml strepromycin sulfate) containing 1 nM insulin for 14-16 hours (18 hours for adenovirus groups) until being used for the indicated experiments. For the treatments, primary hepatocytes were washed once with 4 ml of PBS and then incubated in 2 ml of medium A containing indicated reagents for indicated time as shown in the figure legends.

Cell Transfection and Luciferase Assay

All plasmids were isolated using the Qiagen Plasmid Maxi Kits (Qiagen) and dissolved in 600 μl of water. To extract the plasmids, equal volume of Phenol: Chloroform: Isoamyl Alcohol (25:24:1, v/v) was added and the mixture

was vigorously vortexed for 15 s and spun at 14,000 rpm for 5 min at room temperature (RT). The upper supernatant (~580 µl) was transferred to a new vial and mixed with equal volume of chloroform. The mixture was vigorously vortexed for 15 s and spun at 14,000 rpm for 5 min at RT. The resulted upper supernatant was transferred to a new vial, mixed with 1/10 volume of 3M sodium acetate (pH 5.2) and 2 volumes of absolute ethanol, frozen overnight at -20°C, and spun at 14,000 rpm for 30 min at 4°C. The pellet was washed once with 70% ethanol and dissolved in 500 µl water for assessment of DNA concentration.

Methods for plasmid transfection with primary hepatocytes and INS-1 cells, and dual luciferase assay have been described previously [12, 168]. For transfection of primary hepatocytes, one dish (60 mm) of hepatocytes were washed once with 4 ml of PBS, incubated in 2 ml of serum- and antibiotic-free RPMI medium 1640 at pH 7.55, and co-transfected with 1.8 µg of the respective luciferase reporter construct and 0.1 µg rat RARα, RXRα, and/ or RXRγ expression vectors. Transfection efficiency was monitored by co-transfection with 0.2 µg of *Renilla* luciferase expression vector (pRLSV40)(Promega). Appropriate amounts of the empty expression vectors pGL3-Basic were added to make a total of 2.2 µg of plasmids. Transfections were performed with 10 µl/ dish of Lipofectin (Life Technologies) according to manufacturer's instructions. 6 h post transfection, the cells were washed twice with 4 ml of PBS and incubated in 2 ml of medium containing indicated reagents for 21 h as shown in the figure legends. The cells were then washed once with 4 ml of PBS and incubated in 0.4 ml of 1 × passive lysis buffer (Promega, Madison, WI) on a rocking plates for 20 at RT before they were collected by scraping and stored at -70°C for measurement of luciferase activities.

For transfection of INS-1 cells, one dish (35 mm) of 833/15 cells grown to 70% confluence were washed once with 2 ml of PBS, incubated in 1 ml of serum- and antibiotic-free RPMI medium 1640, and co-transfected with 0.9 µg of the respective luciferase reporter construct and 0.05 µg rat RARα, RXRα,

and/ or RXRγ expression vectors. Transfection efficiency was monitored by cotransfection with 0.1 μg of *Renilla* luciferase expression vector (pRLSV40). Appropriate amounts of the empty expression vectors pGL3-Basic were included to make a total of 1.1 μg of plasmids. Transfections were performed with 10 μl/ dish of Fugene 6 transfection reagent (Roche Applied Science, Indianapolis, IN) according to manufacturer's instructions. 6 h post transfection, the cells were washed twice with PBS and incubated in 1 ml of medium containing indicated reagents for 21 h as shown in the figure legends, and cells were then washed once with 2 ml of PBS and collected by scraping in 0.17 ml of 1 × passive lysis buffer as described above.

Firefly and *Renilla* luciferase activities in the cell lysates were measured with the Dual-Luciferase reporter assay system (Promega) according to the manufacturer's protocol. Photon production was detected as relative light units by using an Orion L Microplate Luminometer (Berthold System, Pforzheim, Germany). All values represent the mean of duplicate transfections, each assayed in triplicate. The amount of firefly luciferase activity in transfected cell lysates was normalized to the amount of *Renilla* luciferase activity from the same test tube. Normalized luciferase activities are shown as the mean ± S.D. of three independent experiments performed in duplicate and are expressed as -fold increases relative to the basal activity of the reporters in the absence of expression plasmids.

Preparation of Recombinant Adenovirus

Methods for recombinant adenovirus generation have been described previously [334]. HEK293 cells were seeded into 6-well plates at a density of 1 × 10^6 cells/well in DMEM supplemented with 10% fetal bovine serum, 100 U/ml penicillin, and 100 μg/ml streptomycin sulfate, and allowed to grow to 90% confluence. The cells were co-transfected with 1 μg each of pACCMV5-RARα, pACCMV5-RXRα, or pACCMV5-HNF4α and pJM17 with Lipofectamine 2000 (Life Technologies) according to manufacturer's instructions. Transfected 293 cells were incubated in 2 ml of DMEM

containing 2% FBS at 37°C and 5% CO_2. Samples demonstrating lysis 6-10 days post-transfection were harvested for viral amplification. Crude lysates were screened for the presence of the specific genes via PCR.

For each recombinant adenovirus, confirmed original crude lysate was then used to infect HEK 293 cells grown to 80% confluence in 150 mm tissue culture plates. The ratio of the medium to crude lysate is 10 to 1 (v/v). After the lysis of the cells at around 48 h post infection, the cell culture medium (crude lysate) was collected, and stored at -80°C until being used.

For purification of each recombinant adenovirus, NP-40 was first added into the crude lysate to reach the final concentration at 0.5%. The mixture was shaken gently at RT for 30 min and subjected to centrifugation at 8,000 rpm and 4°C for 15 min. The supernatant was transferred to a clean bottle, and 0.5 × volume of 20% PEG8000/ 2.5 NaCl was added. The preparation was shaken gently at 4°C overnight. The resulting mixture was transferred to centrifuge bottles and spun at 12,000 rpm at 4°C for 15 min. The precipitated pellet was resuspended in a small volume of PBS (2-3 ml), and spun at 12,000 rpm and 4°C for 10 min to remove insoluble matters. Solid CsCl was added to the supernatant until its final density reached 1.34 g/ml. The mixture was spun at 90,000 rpm at 25°C for 3 h using Optima™ MAX-XP Ultracentrifuge (Beckman Coulter Inc., Brea, CA). The corresponding band containing pure viral particles was collected in a total volume less than 1 ml for desalting. The PD-10 column Sephadex™ G-25 M (Amersham Pharmacia Biotech AB, Sweden) was equilibrated with 5 ml PBS. The purified virus in CsCl solution was loaded onto the column and eluted with 5 ml PBS. The flow through was collected into ten fractions. The optical density (OD) of each of these fractions at 260-nm was determined after 1 to 50 dilution in water using Spectronic® GENESYS™ 5 Spectrophotometer (Thermo Scientific Inc., Pittsburgh, PA). The fractions containing significant values of OD (usually at around fractions 7-9) were collected and pooled. Bovine serum albumin (BSA) and glycerol were added to the pooled solution to make the stock viral solution with the final concentrations of them at 0.2% and 10%, respectively. After the

filtration of the stock solution for sterilization, its OD was determined to estimate the plaque forming units (*pfu*). We used that 1 OD equals to 1×10^{12} *pfu*/ml. The final purified virus stock was frozen at -80°C until being used in the indicated experiments.

Recombinant adenovirus expressing human COUP-TFII (Ad-COUP-TFII) was provided by Mireille Vasseur-Cognet [207].

RNA Preparation and Quantitative Real-Time RCR (qPCR)

Isolation of total RNA and qPCR were performed as described [334]. Rat hepatocytes in 60 mm plates were infected with purified recombinant adenoviruses at 1000 *pfu*/cell for 18 h. After the indicated treatments, total RNA was extracted from the treated cells using 1 ml of RNA STAT 60 reagent (TEL-TEST Inc., Friendswood, TX) according to the manufacture's protocol. The contaminated DNA was removed using the DNA-*free*™ kit (Applied Biosystems) according to the manufacture's instruction. First strand cDNA was synthesized using cDNA synthesis kit (Applied Biosystems). The reaction was carried out in a total volume of 100 µl, containing 2 µg of DNA-free RNA, 50 mM Tris-HCl, 75 mM KCl, 10 mM DTT, 8.5 mM magnesium chloride, 0.5 mM of each dNTP (dATP, dCTP, dTTP, and dGTP), 2.5 mM random hexamer primers, 40 units of RNase inhibitor, 125 units of multiscribe reverse transcriptase. The conditions are 25°C for 10 min, 48°C for 30 min, and 95°C for 5 min. The qPCR primer sequences are summarized in Table 4.2. Each SYBR Green based qPCR reaction contains, in a final volume of 14 µl, cDNA from 14 ng of reverse transcribed total RNA, 2.33 pmol primers, and 7 µl of 2 × SYBR Green PCR Master Mix (Applied Biosystems). Triplicate PCR reactions were carried out in 96-well plates using 7300 Real-Time PCR System (Applied Biosystems). The conditions are 50°C for 2 min, 95°C for 10 min, followed by 40 cycles of 95°C for 15 seconds, and 60°C for 1 min. The gene expression level was normalized to that of invariable control gene *36B4*. Data are presented as the induction fold for which the control group is arbitrarily assigned a value of 1 using the $\Delta\Delta C_T$ method.

Western Blotting

Proteins from primary hepatocytes infected with recombinant adenoviruses were isolated and their concentrations were determined as described [7]. Rat hepatocytes in 60 mm plates were infected with purified recombinant adenoviruses as shown in the figure legends at 1000 *pfu*/cell for 18-24 h, washed once with 4 ml of PBS, and scrapped into 0.4 ml of whole-cell lysis buffer (1% Triton X-100, 10% glycerol, 1% IGEPAL CA-630, 50 mM Hepes, 100 mM NaF, 10 mM EDTA, 1 mM sodium molybdate, 1 mM sodium β-glycerophosphate, 5 mM sodium orthovanadate, 1.9 mg/ml aprotinin, 5 µg/ml leupeptin, 1 mM benzamide, 2.5 mM PMSF, pH 8.0). The lysates were placed on ice for at least 20 min before being subjected to centrifugation at 13,00 rpm for 20 min. The protein concentration in the supernatant was determined with PIERCE BCA protein assay kit (Rockford, IL). Proteins (40 µg/lane) in whole cell lysates were separated on a 10% SDS-polyacrylamide gel, transferred onto BIO-RAD Immuno-Blot PVDF membrane (Hercules, CA), blocked for 1 h at RT in TBS plus 0.05% TWEEN®20 (TBST), 5% non-fat dry milk, and incubated overnight at 4°C in primary antibodies against RARα (# 2554), RXRα (#5388), HNF4α (#3113), COUP-TFII (#6434), or β-Actin (#4970S) diluted 1:1000 in TBST and 1% BSA. Blots were washed with TBST twice for 5 min, twice for 10 min at RT, and incubated for 1 h at RT in secondary antibody anti-rabbit IgG horseradish peroxidase (#7074P2) diluted 1:5000 in TBST. Blots were then washed with TBST twice for 5 min and twice for 10 min at RT. All blots were developed using the ECL Western blotting system (Thermo Scientific) and exposed to X-ray films (Phenix Research Products, Candler, NC) for protein band detection. The films were scanned using an HP Scanjet 3970 (Palo Alto, CA) and stored as Tagged Image File Format.

Table 4.2 Primers for qPCR

Gene	Primer (oGC-)	Sequence (5'-3')
36B4		TTCCCACTGGCTGAAAAGGT
		CGCAGCCGCAAATGC
Gck	222	CCTGGGCTTCACCTTCTCCTT
	223	GAGGCCTTGAAGCCCTTGGT
Pck1		AGTCACCATCACTTCCTGGAAGA
		GGTGCAGAATCGCGAGTTG
Srebp-1c		GGAGCCATGGATTGCACATT
		AGGCCAGGGAAGTCACTGTCT
Igfbp1	636	GATCACTGACCTCAAGAAATGGAA
	637	GCGGCACGTAATCTCTCTAACA
Cyp26a1	452	AGTGATGGGCGCGGATAAT
	453	TGCACTGACACCAACCGGT
Apociii	1081	GAACAAGCCTCCAAGACGGT
	1082	GGGATTTGAAGCGATTGTCC
Rara	580	GTCATCCGGCTACCACTATGG
	581	TGGATACTGCGTCGGAAGAAG
Rarb	582	GGCCTCTGGGACAAATTCAG
	583	GCAGACGCTTGGCGAACT
Rxra	654	GCCCAAATGACCCAGTGACT
	655	TCGTCCAGAGGTAGGGAGGAA
Rxrg	658	TGCGGATAAGCAGCTCTTCA
	659	TCCAAGGTGAGGTCTGAGAAGTG
Hnf4a	998	CAAGAACACATGGGCACCAATG
	999	GGTGATGGCTGTGGAGTCT

Table 4.2 Continued.

Gene	Primer (oGC-)	Sequence (5'-3')
Coup-tfii	1057	TGCCTGTGGTCTGTCTGATG
	1058	GGAAGGGAGACGAAGCAAAA
Gck promoter	968	GGGTGGCTCCTGAAATACCC
	969	CCTCATGCCTACCTGTTTCG

Preparation of Nuclear Extracts

Nuclear extracts were prepared from rat liver essentially as described previously [335]. All manipulations were performed in the cold, and all solutions, tubes, and centrifuges were chilled to 0°C. When included, phenylmethylsulfonyl fluoride (PMSF), spermidine, and dithiothreitol (DTT) were added to the buffers just prior to use. Minced rat liver (~10 g) was brought up to 30 ml with homogenization buffer (10 mM Hepes-KOH [pH 7.8], 10 mM KCl, 0.1 mM EDTA, 1 mM spermidine, 1mM DTT, 0.5 mM PMSF, 0.3 M sucrose) and was homogenized using a 100 ml Wheaton glass homogenizer until more than 90% of the cells were broken. The homogenate was diluted with one-third volume (~ 10 ml) of cushion buffer (20 mM Hepes-KOH [pH 7.8], 25 mM KCl, 0.5 mM EDTA, 0.5 mM EGTA, 1 mM spermidine, 1 mM DTT, 0.5 mM PMSF, 2 M sucrose, 10% glycerol). The resulted mixture was aliquoted into six 6-ml portions, each of which was then layered onto 2 ml cushion buffer, and centrifuged at 24,000 rpm for 1 h at 4°C in an Sorvall TH-64I rotor (Sorvall Ultra-80). The nuclear pellets were resuspended in 2 ml lysis buffer (10 mM Hepes-KOH [pH 7.8], 100 mM KCl, 3 mM $MgCl_2$, 1 mM DTT, 10% glycerol, 0.5 mM phenylmethylsulfonyl fluoride, 1.9 µg/ml aprotitin, 1 µM pepstatin A, 5 µg/ml leupeptin, 1 mM sodium beta-glycerol phosphate, 1 mM sodium molybdate, 1 mM benzamidine, 5 mM sodium metavanadate, and 5 mM sodium fluoride), and centrifuged as described above. The pelleted nuclei were resuspended in 0.5 ml of lysis buffer and combined. One-tenth volume of 5 M $(NH_2)SO_4$ was added dropwise, and the extract was gently shaken for 1 h at 4°C. The viscous lysate was then centrifuged at 90,000 rpm for 1 h at 4°C in a TLA 100.3 rotor (Optima^{TM} Max-XP Ultracentrifuge, Beckman Coulter) to pellet chromatin. The supernatant was aliquoted and stored at -70°C. Once thawed, the extracts were not refrozen.

INS-1 833/15 cells grown to 80% confluence in 150 mm plates were infected with recombinant adenoviruses Ad-RARα, Ad-RXRα, Ad-HNF4α, Ad-COUP-TFII or control Ad-β-gal at 1000 *pfu*/cell for 24 h, washed once with 25 ml of PBS, scrapped into 1 ml of homogenization buffer, and transferred into a

1 ml Wheaton glass homogenizer. A tight-fit pestle was passed up and down for 120 times (30 strikes each for 4 times). The lysate was spun at 1000 × g for 10 m. The pellet was suspended into 0.67 ml of homogenization buffer and mixed with 1.33 ml of cushion buffer. The resulted 2 ml mixture was over-layered onto 1 ml of cushion buffer in a 14 × 89 mm centrifuge tube (Beckman), and spun at 24,000 rpm for 1 h. The pellet at the bottom of the cushion buffer was suspended in 100 µl of nuclear extract lysis buffer and transferred to a micro centrifuge tube. The suspended nuclei were mixed with 0.1 volume of 5 M NaCl and rotated at 4°C for 30 m. After a 14,000 rpm spin to pull down the nuclear lysate, it was transferred to a centrifuge tube and spun at 85,000 rpm for 45 m. The resulted unclear extract was stored at -70°C before protein content determination and further analysis as indicated in the figure legends.

Electrophoretic Mobility Shift Assay (EMSA)

All oligonucleotides used for EMSAs were summarized in Table 4.3. Double-stranded synthetic oligonucleotide probes were prepared by heating equal molar amounts (100 pmol) of complementary synthetic oligomers in a volume of 100 µl containing 50 mM NaCl to 100°C for 10 min, then allowing the hybridization mix to cool to ambient temperature. The resulting double-stranded fragments were labeled by 5'-end labeling with [γ-^{32}P] ATP (3000 mCi/mmol, Perkin Elmer, Waltham, MA) and T4 polynucleotide kinase (PNK, New England Biolabs, Ipswich, MA) in a total volume of 20 µl, containing 2 pmol probes, 70 mM Tris-HCl (pH 7.6), 10 mM $MgCl_2$, 5 mM DTT, 10 units of PNK, and 5 µl [γ-^{32}P] ATP. After incubation at 37°C for 15 min, the reaction was terminated by addition of EDTA to a final concentration of 10 mM. Labeled fragments were purified with Illustra MicroSpin G-50 columns (GE healthcare, Pittsburgh, PA).

Binding reactions were carried out in a total volume of 20 µl, containing 10 mM Hepes-KOH (pH 7.9), 100 mM KCl, 5 mM $MgCl_2$, 0.5 mM EDTA, 2 mM dithioerythritol, 100 ng/µl poly d(I-C), 5% (v/v) glycerol, 100 ng/µl bovine

serum albumin and 6 µg nuclear extract. After preincubation for 10 min at RT, 10^5 c.p.m. labeled probe was added and the incubation was continued for an additional 20 min at RT.

In Figure 4.11 and 4.12, 1 µg of each of the specific antibodies or a combination of them, together with appropriate amounts of IgG control were added to preincubated DNA-protein complexes for 20 m at RT before addition of probe. The electrophoresis was then performed with a 12% (w/v) non-denaturing polyacrylamide gel (acrylamide/ bis-acrylamide ratio 37.5:1) in 0.5 × TBE beffer (44.5 mM Tris/ 44.5 mM boric acid/ 1 mM EDTA) at constant current 10 mA for 5 h at 4°C. After electrophoresis the gels were dried using Slab Gel Dryer (Model SE 540, Hoefer Scientific Instruments, San Francisco, CA) that is connected to a Savant Refrigerated Condensation Trap (Thermo Scientific). Gels were then exposed to X-ray films (Phenix Research Products) at RT for the time indicated in figure legends. The films were scanned using an HP Scanjet 3970 and stored as Tagged Image File Format.

Chromatin Immunoprecipitation (ChIP) Assay

The ChIP assay was performed based on a previously described method with some modifications [336]. Following the treatment as indicated in the figure legends, hepatocytes or HL1C cells in 150 mm plates were washed twice with 25 ml of PBS and cross-linked in 10 ml of PBS by adding formaldehyde to a final concentration of 1% and incubating for 15 min at 37°C. The cross-linking reaction was quenched by adding one-ninth volume of 1.25 M glycine and continued the incubation for 5 min at RT. Cross-linked cells then were washed with 25 ml of ice-cold PBS twice, scrapped into 1 ml of ice-cold PBS, and centrifuged for 5 min at 2000 × g. Cells were then resuspended in 600 µl of lysis buffer (1% SDS, 10 mM Tris-HCl [pH 8], 10 mM EDTA, 0.5 mM phenylmethylsulfonyl fluoride, 1.9 µg/ml aprotitin, 1 µM pepstatin A, 5 µg/ml leupeptin, 1 mM sodium beta-glycerol phosphate, 1 mM sodium molybdate, 1 mM benzamidine, 5 mM sodium metavanadate, and 5 mM sodium fluoride), incubated for 30 min at 4°C, and sonicated with 45 pulses of

Table 4.3 Oligonucleotides for EMSA

Oligonucleotides	**Primer (oGC-)**	**Sequence (5'-3')**
EMSA-Gck-m6/7-WT	1010	GGCCCTGGCCCTGACCTTGTGACACTAG
	1011	CCTGCCTAGTGTCACAAGGTCAGGGCCA
EMSA-Gck-m6/7-mut	1012	GGCCCTGGCCCTGAatTcGTGACACTAG
	1013	CCTGCCTAGTGTCACgAatTCAGGGCCA
EMSA-Gck-m6/7-4-1	1020	GGCCCTGGCCCTGtCCTTGTGACACTAG
	1021	CCTGCCTAGTGTCACAAGGaCAGGGCCA
EMSA-Gck-m6/7-4-2	1022	GGCCCTGGCCCTGAgCTTGTGACACTAG
	1023	CCTGCCTAGTGTCACAAGcTCAGGGCCA
EMSA-Gck-m6/7-4-3	1024	GGCCCTGGCCCTGACaTTGTGACACTAG
	1025	CCTGCCTAGTGTCACAAtGTCAGGGCCA

10 s each at power setting 60% with the Misonix Sonicator 4000 Ultrasonic Liquid Processor (Qsonica, Newtown, CT) to fragment 300 bp to 500 bp. The resulted mixture was centrifuged for 10 min at 12,000 rpm. Supernatants were collected and diluted with ten volumes of dilution buffer (0.01% SDS, 1% Triton X-100, 1 mM EDTA, 10 mM Tris-HCl [pH 8], 150 mM NaCl). One-tenth volume was saved as 'total input' in the subsequent DNA extraction and PCR analysis. The rest was divided into 2 portions (hepatocytes) or 4 portions (HL1C cells) and each was incubated with 2 µg IgG control (sc-2027), or antibody against RAR (sc-773X), RXR (sc-774X), HNF4α (sc-8987X), or COUP-TFII (PP-H7147-00). After the overnight incubation at 4°C with gentle rotation, 20 µl Dynabeads® protein A (Life Technologies, Norway) and 100 µg/ml sheared salmon sperm (Invitrogen) DNA were added and the incubation continued for 2 h at 4°C. The tube was then placed on a magnetic separation stand to collect the Dynabeads protein A-Ig complex at the tube wall. The immune complexes were washed sequentially for 10 min each in TSE I (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl [pH 8], 150 mM NaCl), TSE II (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl [pH 8], 500 mM NaCl), and buffer III (0.25 M LiCl, 1% NP-40, 1% deoxycholate, 1 mM EDTA, 10 mM Tris-HCl [pH 8]). The immune complexes were then extracted twice with 150 µl freshly prepared elution buffer (1% SDS, 0.1 M $NaHCO_3$) for 20 min at 4°C with gentle rotation. To reverse the crosslinking, 1 µl RNase A (10 mg/ml) was added to the eluates and incubated at 65°C for at least 5 h. To precipitate the DNA, 1/10 volume of 3M sodium acetate (pH5.2), 2.5 volumes of cold 100% ethanol, and 10 mg of glycogen were added, incubated overnight at -20°C, and centrifuged for 20 min at 12,000 rpm. To remove protein, 100 µl Tris-HCl/EDTA (TE, pH 8.0) and 2 µl proteinase K (10 mg/ml) were added and incubated for 1.5 h at 55°C. DNA was extracted once with Phenol: Chloroform: Isoamyl Alcohol (25: 24: 1, v/v) and resuspended in 50 µl TE. 2 µl of DNA were amplified by PCR for a 251 bp hepatic *Gck* promoter region between nucleotide -390 and nucleotide -140 (relative to the translation initiation site +1), using primer pair oGC968/969, under 35 cycles of 94°C for

30 s, 59°C for 45 s, and 72°C for 1 min. The PCR products were resolved on 2% agarose gels containing ethidium bromide.

Statistical Analysis

Quantitative results are expressed as means ± standard edeviation (SD). Levene's test was used to determine homogeneity of variance among groups using SPSS 21 statistical software (IBM, Armonk, NY) and where necessary natural log transformation was performed before analysis. We performed the statistical analyses using one-way analysis of variance (ANOVA) when more than two groups of data were compared, and Student's *t* test when only two groups of data were concerned. We considered the difference to be statistically significant at $p < 0.05$.

4.3 Results

Progressive Deletion of 5' Flanking Region of Rat Hepatic *Gck* Promoter

Transient transfection studies of rat hepatocytes revealed that a reporter plasmid with 3.4 kb of genomic DNA flanking the liver *Gck* promoter elicits the maximal luciferase activity. In addition, the gene fragment from -1003 to -707 (with respect to the start of transcription) was shown to be a bona fide hepatocyte-specific enhancer [92]. In our preliminary observations, luciferase reporter gene construct containing about 1 kb (with respect to the start of translation) of rat *Gck* promoter is sufficient to mediate RA response. Therefore, a series of luciferase gene reporter constructs containing deletions of this 1 kb promoter fragment were constructed. As shown in Fig. 4.1, plasmids A, B, C, D, and E contain about 1, 0.8, 0.6, 0.4, and 0.2 kb of 5' flanking region of liver *Gck* promoter, respectively. Primary hepatocytes were transfected with reporter plasmids, followed by exposure to RA (5 μM), insulin (INS, 10 nM), or both for 21 hours, and luciferase activity was subsequently analyzed. RA and RA + INS stimulated the luciferase activity of plasmid A by 1.9- and 2.3-fold, respectively. Progressive deletion of promoter did not result

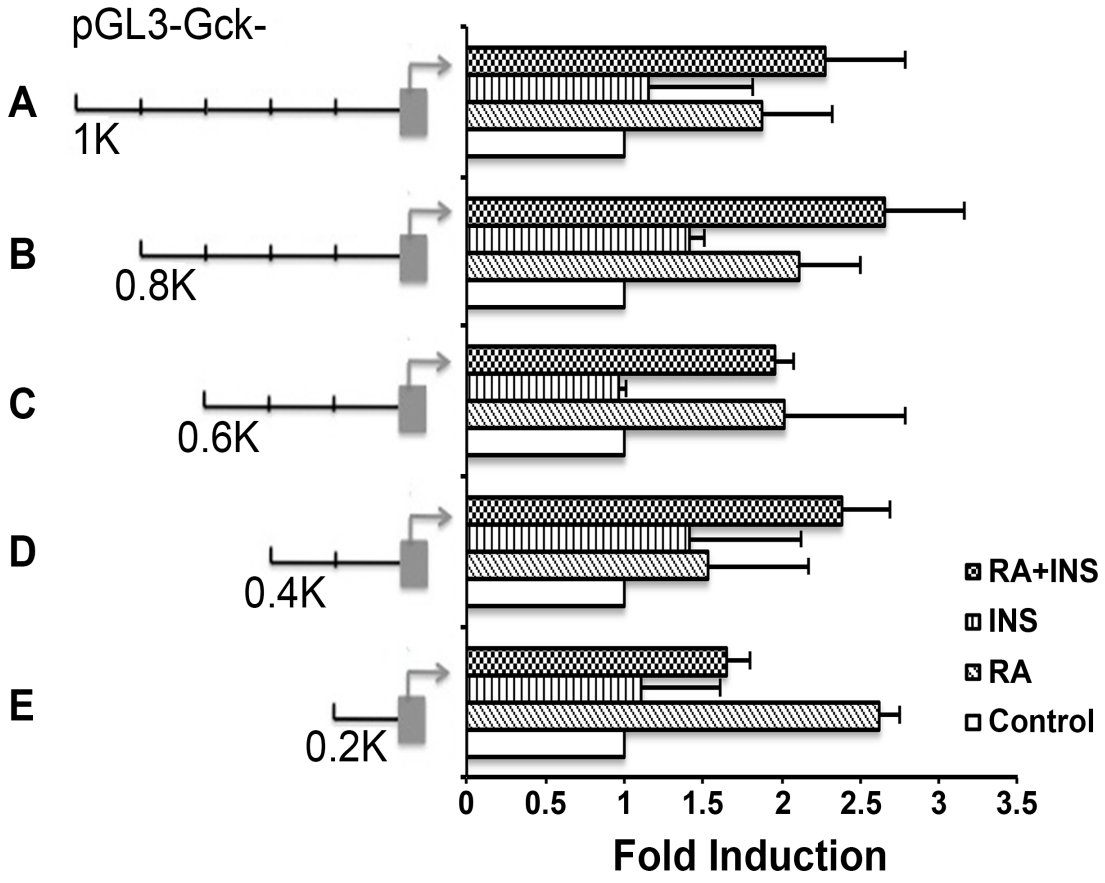

Figure 4.1 Deletion analysis of rat hepatic *Gck* promoter in rat primary hepatocytes

Primary rat hepatocytes were transfected with 2 μg of indicated constructs together with 0.2 μg phRL4-TK as transfection efficiency control using Lipofectin as described in *Materials and Methods*. Six hours after transfection, the medium was switch to medium without or with 10 nM insulin in the absence or presence of 5 μM RA and incubated for 21 h, after which the cells were harvested and assayed for dual luciferase activities as described in *Materials and Methods*. Each value represents the mean ± SD of three independent transfection experiments in duplicate. The luciferase activity of each promoter in the presence of control (ethanol) was arbitrarily assigned a value of 1.

in decrease in luciferase expression, as demonstrated by similar fold induction of plasmids B-E under the same conditions. Insulin had little effect on any of the plasmids and there was no significant difference between RA and RA + INS group. These results suggest that the RAREs may be located in the promoter fragment in plasmid E.

Over-expression of RARα Augments RA Response of Reporter Gene Constructs

Given the moderate induction in luciferase activity of plasmids A-E by RA, we next tried to test whether we can enhance the RA induction further in case that multiple RAREs exist in *Gck* promoter. This prompted us to test whether over-expression of RARs and/or RXRs is able to augment RA-induced luciferase activity and re-introduce insulin response of reporter plasmids. As shown in Fig. 4.2, transient co-transfection of plasmid A and pGL3 empty vector into rat primary hepatocytes resulted in 1.8- and 2.2-fold induction of luciferase activity by RA and RA + INS, respectively. Surprisingly, over-expression of RARα, but not RXRα or RXRγ, substantially augmented the RA- and RA + INS-mediated increase in luciferase activity to 4.1- and 5.5-fold, respectively. Co-expression of RARα and RXRα or RXRγ did not further increase the fold induction. Over-expression of these retinoid receptors had little effect on basal levels of transcription. These data together with those described above suggest that RA response of proximal *Gck* promoter can be observed in the absence of insulin stimulation and that primary hepatocytes are not fungible in the identification of RARE in *Gck* promoter.

Reconstitution of RA Response of *Gck* Reporter Constructs in INS-1 Cells

Due to the persistent RA-induced *Srebp-1c* expression in INS-1 cells, they were used successfully to identify RARE in *Srebp-1c* promoter [12]. To evaluate if INS-1 833/15 cell line would be an appropriate tool for identification of RARE in *Gck* promoter, we repeated the transient transfection experiments

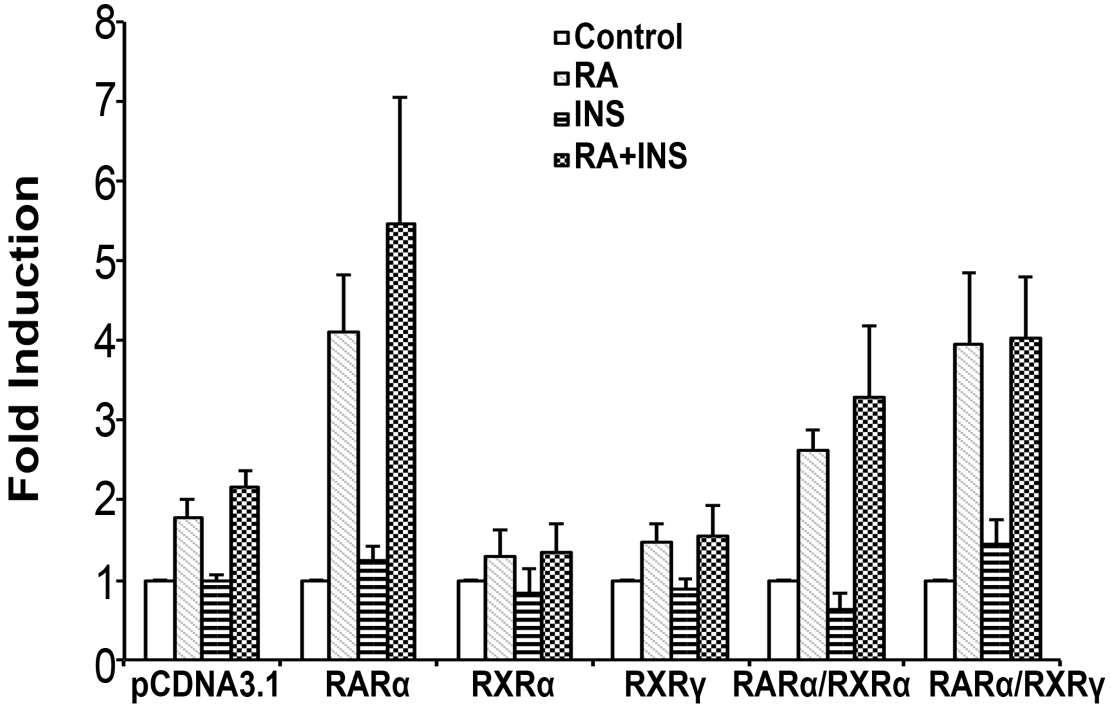

Figure 4.2 Activation of plasmid A in rat primary hepatocytes

Primary rat hepatocytes were transfected with 2 μg of plasmid A together with 0.2 μg phRL4-TK as transfection efficiency control and 0.2 μg expression vectors as indicated using Lipofectin. 6 hours after transfection, the medium was switch to medium without or with 10 nM insulin in the absence or presence of 5 μM RA and incubated for 21 h, after which the cells were harvested and assayed for dual luciferase activities as described in *Materials and Methods*. Each value represents the mean ± SD of three independent transfection experiments in duplicate. The luciferase activity of promoter in the presence of control (ethanol) was arbitrarily assigned a value of 1.

in 833/15 cells. Similar observations were obtained from INS-1 833/15 cells. Thus, 833/15 cells were transiently co-transfected with plasmids A, C, D, E, or pGL3 empty vector, together with pcDNA3.1 empty vector, pcDNA3.1-RARα, pcDNA3.1-RXRγ, or pcDNA3.1-RARα + pcDNA3.1-RXRγ (Fig. 4.3). Luciferase activities were measured after incubation of the cells for 21 h in the presence or absence of 5 μM RA. For plasmid A, over-expression of RARα slightly but significantly increased the level of basal transcription. Over-expression of RXRγ alone did not change the basal transcription. However, co-expression of RARα and RXRγ resulted in 2.3-fold induction of basal transcription. Plasmid C, D, and E showed the same level of basal transcription under all conditions. With the expression of RARα, but not RXRγ, RA induced 7.3-, 6.7-, and 6,5-fold activation of deletion constructs A, C, and D, respectively. The combination of RARα and RXRγ resulted in more robust induction of RA-dependent promoter activity, which are 12.2-, 10.6-, and 11.5-fold, respectively. Luciferase activity of plasmid E was still substantially activated by RA both with RARα and RARα + RXRγ (4.3- and 6.7-fold, respectively). However, the folds were significantly less than other reporter constructs, suggesting that there might be more than one RARE in the hepatic *Gck* promoter, one located within the 0.2 kb region (plasmid E) for sure and possibly the other between 0.4 kb and 0.2 kb (plasmid D).

Linker-scan Analysis of 0.2 kb Hepatic *Gck* Promoter Region in Plasmid E

To identify the possible RARE in the 0.2 kb region of plasmid E, we introduced a series of overlapping 12-bp scramble mutations covering the nucleotide sequence from -237 to -118 into the 0.2 kb deletion promoter construct to generate plasmids m1 to m12 (Fig. 4.4), which were then co-transfected into primary rat hepatocytes together with pcDNA3.1-RARα and pcDNA3.1-RXRγ. Promoter activity of the WT 0.2 kb (plasmid E) and mutant constructs m1 to m12 in the absence or presence of 5 μM RA was analyzed. The mutations in mutant plasmids m3, m4, m5, and m8 significantly reduced the RA-induced promoter activity. The scramble mutations in m6 and m7

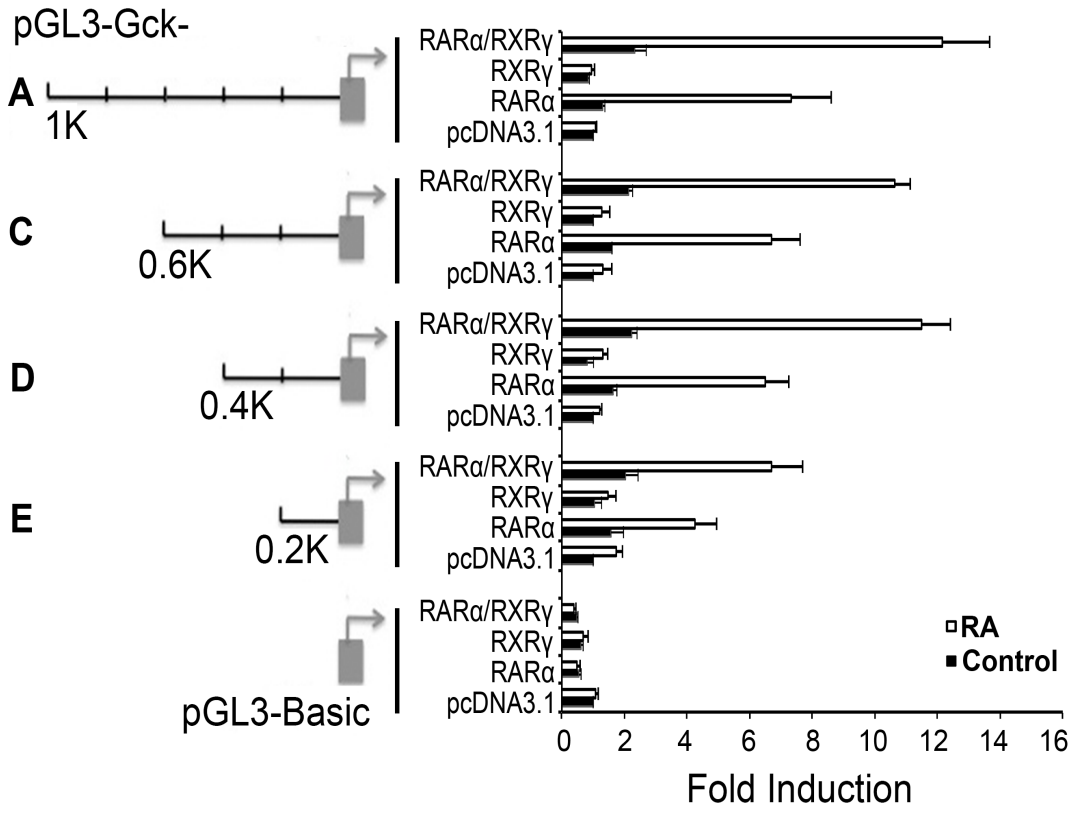

Figure 4.3 Effects of RA on activation of *Gck* reporter gene constructs in INS-1 833/15 cells

INS-1 Cells were transefected with 2 µg of indicated constructs together with 0.2 µg phRL4-TK and 0.2 µg expression vectors using Fugene 6 as described in *Materials and Methods*. Four hours after transfection, the medium was switched to serum free medium without or with 5 uM RA and incubated for 21 hours, after which the cells were harvested and assayed for dual luciferase activities as described in *Materials and Methods*. Each value represents the mean ± SD of three independent transfection experiments in duplicate. The luciferase activity of each promoter co-transfected with pcDNA3.1 in the absence of RA was arbitrarily assigned a value of 1.

Figure 4.4 Nucleotide sequences of wild type (WT) and mutant rat hepatic *Gck* promoters

The sequence of a portion of the normal *Gck* promoter (-237/-118, with respect to the translation initiation start site) is shown at the top. Below the sequence of the WT promoter are shown 12 bp overlapping scramble mutations that are separately introduced into the DNA by site-directed oligonucleotide mutagenesis as described in *Materials and Methods*. The names of the mutant plasmids, m1-m12, are denoted on the left. The mutant scrambled sequence that was introduced is shown below the normal promoter sequence.

completely abolished the RA-activated transcription (Fig. 4.5). Same results were obtained from transfection studies performed with 833/15 cells. The mutants m5 and m8 reduced, while mutants m6 and m7 abolished the RA-induced promoter activity (Fig. 4.6).

Introduction of Scramble Mutations of m6 and m7 into Plasmid A Destroys its RA Response

We next investigated whether there is another RARE located between the 0.4 kb and 0.2 kb regions. To test this, we introduced the scramble mutations in m6 and m7 into plasmid A by replacing the WT nucleotide sequence from -237 to -4 of the 1 kb fragment in plasmid A with corresponding mutant nucleotide sequence in m6 and m7 to get plasmid pGL3-GK-1k-m6 and pGL3-GK-1k-m7, respectively. If the hypothesis that another RARE is present between 0.4 kb and 0.2 kb region is true, these two plasmids should be able to respond to RA. However, introduction of the mutation either in m6 or in m7 destroyed hepatic *Gck* transcription activated by RA, RAR agonist TTNPB, or RXR agonist LG268, or the combination of both (Fig. 4.7), indicating the uniqueness of the RARE located within the 0.2 kb region (plasmid E).

Linker-scan Analysis of *Gck* Promoter Region in Plasmid E Covered by Scramble Mutations of m6 and m7

To map the RARE more precisely in plasmid E, we introduced 5 overlapping 5- to 6- bp scramble mutations covered by scramble mutations in m6 and m7 (-189/-168) into WT plasmid E to generate plasmids m6/7-1, -2, -3, -4, and -5, respectively. Fig. 4.8 shows that mutations in m6/7-4 (-177/-173) resulted in a 54% decrease in RA-dependent activation, suggesting the important role of this ACCTT sequence. Further, point mutations at -177 (A to t), -176 (C to g), and -175 (C to a) impaired the RA-dependent activation of luciferase activities by 39%, 47%, and 39%, respectively, whereas point mutations at -174 (T to C) and -173 (T to A) had little effect on RA responsiveness of reporter constructs (Fig. 4.9). These data suggested that

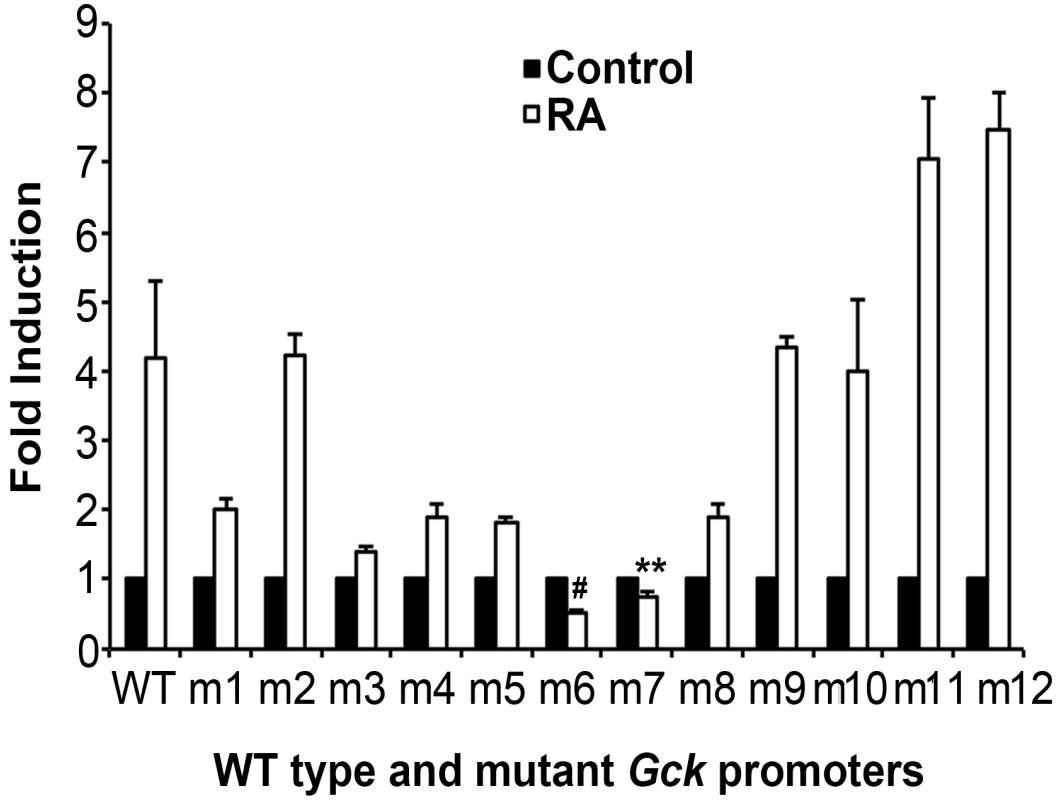

Figure 4.5 Effects of RA on activation of WT and mutant *Gck* reporter gene constructs in primary rat hepatocytes

Primary rat hepatocytes were transfected with 2 μg of indicated plasmids together with 0.2 μg phRL4-TK as transfection efficiency control, 0.1 μg expression vector containing RARα, and 0.1 μg expression vector containing RXRγ using Lipofectin. Six hours after transfection, the medium was switch to medium without or with 5 μM RA and incubated for 21 h, after which the cells were harvested and assayed for dual luciferase activities as described in *Materials and Methods*. Each value represents the mean ± SD of three independent transfection experiments in duplicate. The luciferase activity of WT promoter in the absence of RA was arbitrarily assigned a value of 1 (** $p < 0.01$ and # $p < 0.001$ for comparing control and RA groups).

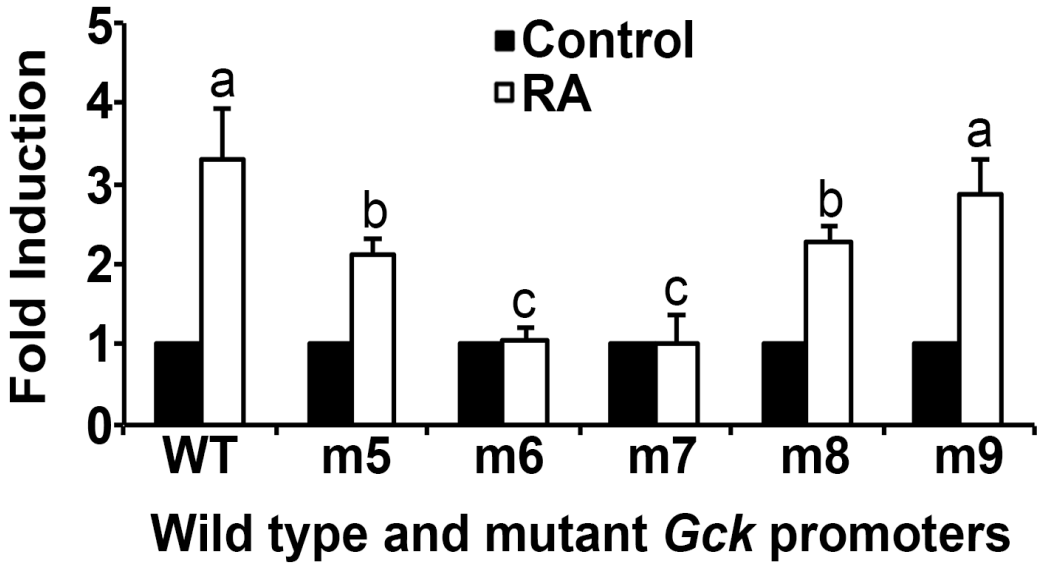

Figure 4.6 Effects of RA on activation of WT and mutant *Gck* reporter gene constructs in INS-1 833/15 cells

Cells were transfected with 2 μg of indicated constructs together with 0.2 μg phRL4-TK, 0.1 μg expression vector containing RARα, and 0.1 μg expression vector containing RXRγ using Fugene 6 as described in *Materials and Methods*. Four hours after transfection, the medium was switched to serum free medium without or with 5 uM RA and incubated for 21 hours, after which the cells were harvested and assayed for dual luciferase activities as described in *Materials and Methods*. Each value represents the mean ± SD of three independent transfection experiments in duplicate. The luciferase activity of each promoter in the absence of RA was arbitrarily assigned a value of 1 ($a > b > c$ for comparing RA groups using one way ANOVA; all $p < 0.05$).

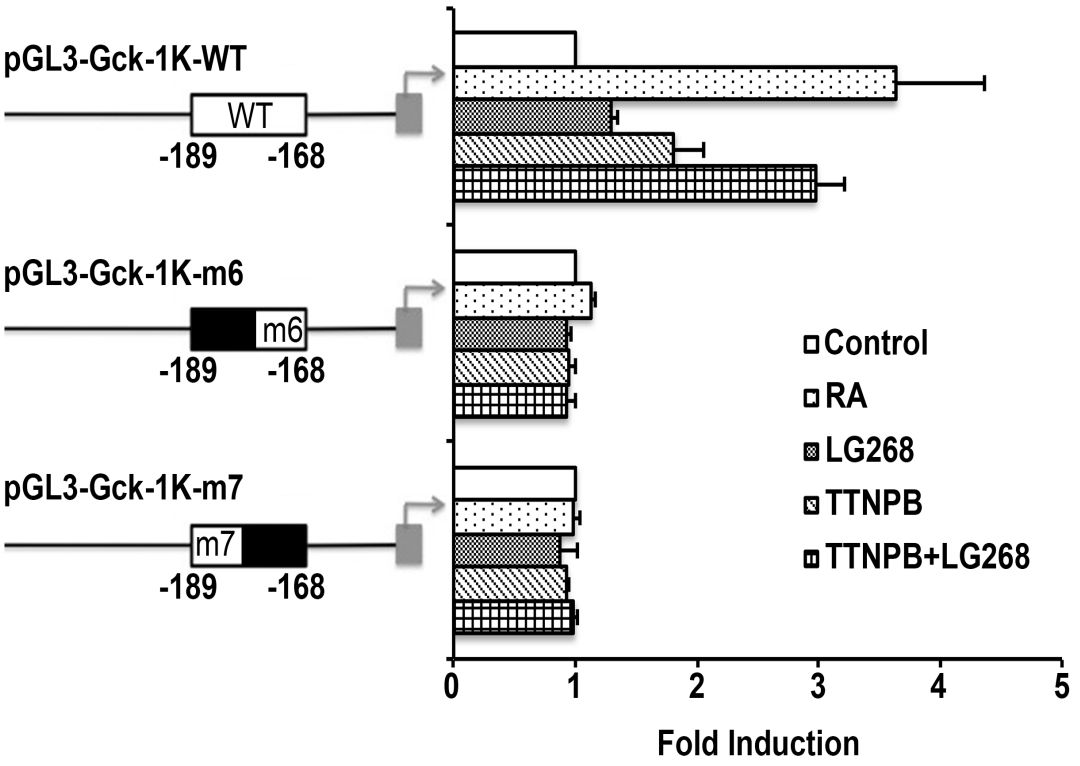

Figure 4.7 Effects of ligands on activation of WT and mutant plasmid A in INS-1 833/15 cells

Plasmid A was digested with *Kpn*I and *Pml*I to yield an approximately 770 bp fragment, which was inserted in the sense orientation of plasmids m6 and m7 cut with *Kpn*I and *Pml*I to obtain pGL3-*Gck*-1k-m6 and pGL3-*Gck*-1k-m7, respectively, as described in *Materials and Methods*. Cells were transfected with 2 μg of indicated constructs together with 0.2 μg phRL4-TK, 0.1 μg expression vector containing RARα, and 0.1 μg expression vector containing RXRγ using Fugene 6 as described in *Materials and Methods*. Four hours after transfection, the medium was switched to serum free medium without or with 5 uM RA and incubated for 21 hours, after which the cells were harvested and assayed for dual luciferase activities as described in *Materials and Methods*. Each value represents the mean ± SD of three independent transfection experiments in duplicate. The luciferase activity of each promoter in the presence of C (control, ethanol) was arbitrarily assigned a value of 1.

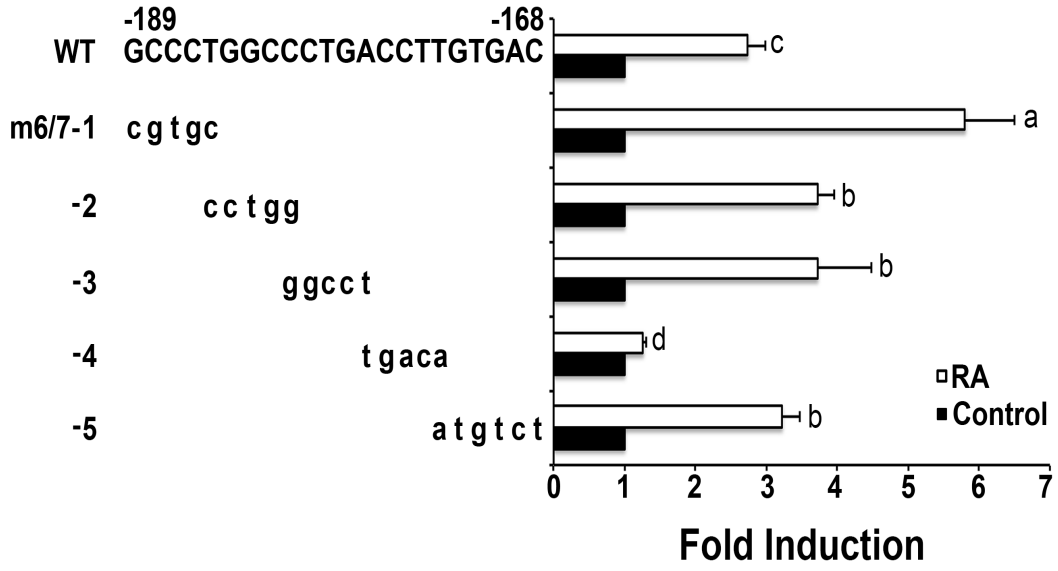

Figure 4.8 Linker-scan analysis of *Gck* promoter region in plasmid E covered by scramble mutations of m6 and m7

(Left) The sequence of WT *Gck* promoter covered by m6 and m7 is shown at the top and numbered as previously described. Five overlapping scramble mutations were separately introduced into the DNA by site-directed oligonucleotide mutagenesis to generate m6/7-1, -2, -3, -4, and -5, respectively, as described in *Materials and Methods*. **(Right)** Fold induction of WT and mutant m6/7 constructs in 833/15 cells. Cells were transfected with 2 μg of indicated constructs together with 0.2 μg phRL4-TK, 0.1 μg expression vector containing RARα, and 0.1 μg expression vector containing RXRγ using Fugene 6 as described in *Materials and Methods*. Four hours after transfection, the medium was switched to serum free medium without or with 5 uM RA and incubated for 21 hours, after which the cells were harvested and assayed for dual luciferase activities as described in *Materials and Methods*. Each value represents the mean ± SD of three independent transfection experiments in duplicate. The luciferase activity of each promoter in the absence of RA was arbitrarily assigned a value of 1 ($a > b > c > d$ for comparing RA groups using one way ANOVA; all $p < 0.05$).

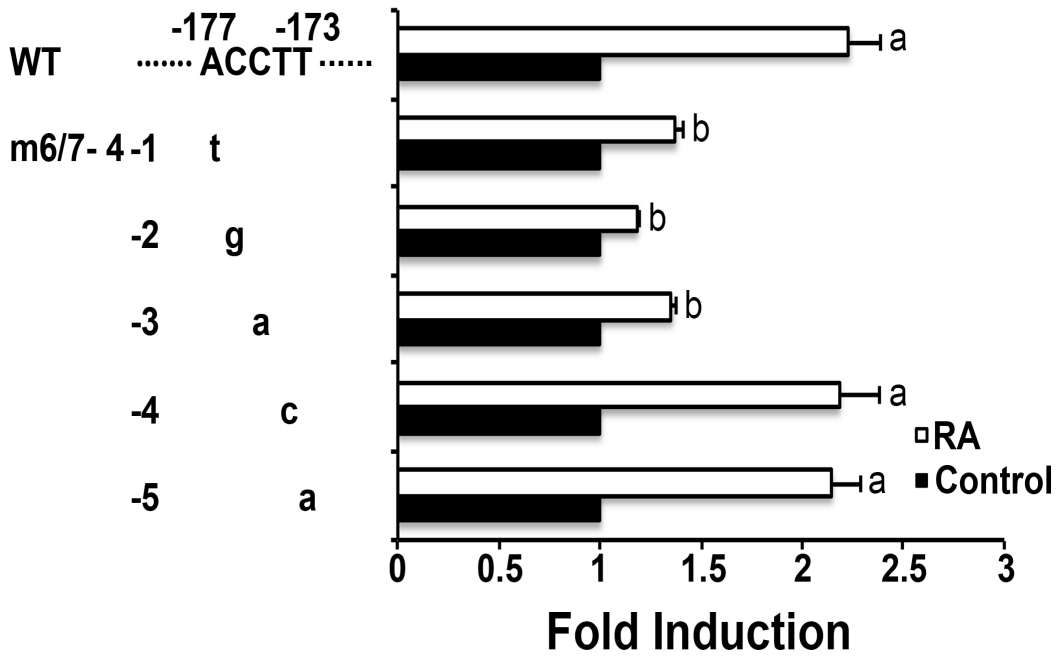

Figure 4.9 Effect of RA on activation of WT and mutant m6/7-4 constructs

(Left) The sequence of WT *Gck* promoter covered by scramble mutation of m6/7-1 is shown at the top and numbered as previously described. Five point mutations were separately introduced into the DNA by site-directed oligonucleotide mutagenesis to generate m6/7-4-1, -2, -3, -4, and -5, respectively, as described in *Materials and Methods*. **(Right)** Fold induction of WT and mutant m6/7 constructs in 833/15 cells. Cells were transfected with 2 µg of indicated constructs together with 0.2 µg phRL4-TK, 0.1 µg expression vector containing RARα, and 0.1 µg expression vector containing RXRγ using Fugene 6 as described in *Materials and Methods*. Four hours after transfection, the medium was switched to serum free medium without or with 5 uM RA and incubated for 21 hours, after which the cells were harvested and assayed for dual luciferase activities as described in *Materials and Methods*. Each value represents the mean ± SD of three independent transfection experiments in duplicate. The luciferase activity of each promoter in the absence of RA was arbitrarily assigned a value of 1 ($a > b$ for comparing RA groups using one way ANOVA; all $p < 0.05$).

RA activated hepatic *Gck* transcription through transcriptional factors bound to the -177/-175 region of its promoter.

Promiscuous Binding of RAR, RXR, HNF4, and COUP-TFII to RARE in Rat Hepatic *Gck* Promoter

Fig. 4.10 shows the alignment of human, rat, and mouse downstream *Gck* promoter DNA sequence (-237/+1) and the regions with high conservation across these three mammalian species. The RARE identified in this study is also a potential HNF4 binding site reported previously [130]. To corroborate the interaction of RAR/RXR with RARE, the binding of proteins in the rat liver nuclear extracts (NEs) to this region of the downstream *Gck* promoter was examined by electrophoretic mobility shift assay (EMSA). An oligonucleotide probe corresponding to the rat *Gck* -190/-163 region (EMSA-*Gck*-m6/7-WT) was able to form five complexes (Fig. 4.11, lane 1, arrows A to E). Mutations of CCTT in the putative RARE/HNF4α binding site to ATTC (EMSA-*Gck*-m6/7-mut) resulted in complete loss of the formation of these binding complexes (Fig. 4.11, lanes 2, 7,12). Single nucleotide mutation in plasmids p*Gck*-1k-m6/7-4-1 (C to A at -177) and p*Gck*-1k-m6/7-4-2 (T to C at -176) had little effect on the formation of the binding complexes. However, point mutation in plasmid p*Gck*-1k-m6/7-4-3 (T to C at -175) enhanced the formation of complexes. To determine the presence of RARs, RXRs, HNF4α, and COUP-TFII in these complexes, individual antibodies against each of them, or in combinations, were included in the binding reaction. Addition of the HNF4α antibody in the binding reaction increased the formation of complex C but decreased the formation of complexes B, D, and E, with EMSA-*Gck*-m6/7-WT probe (Fig. 4.11 and Fig. 4.12). COUP-TFII antibody strongly reduced the formation of complex A (Fi.g 4.11 and Fig. 4.12, lanes 3, 6, 9,10, 12, 13, 15, and 16). The formation of binding complexes seemed not to be affected by addition of RAR or RXR antibody alone (Fig. 4.12, lanes 4 and 5, respectively).

To further confirm these observations, NEs were prepared from INS-1

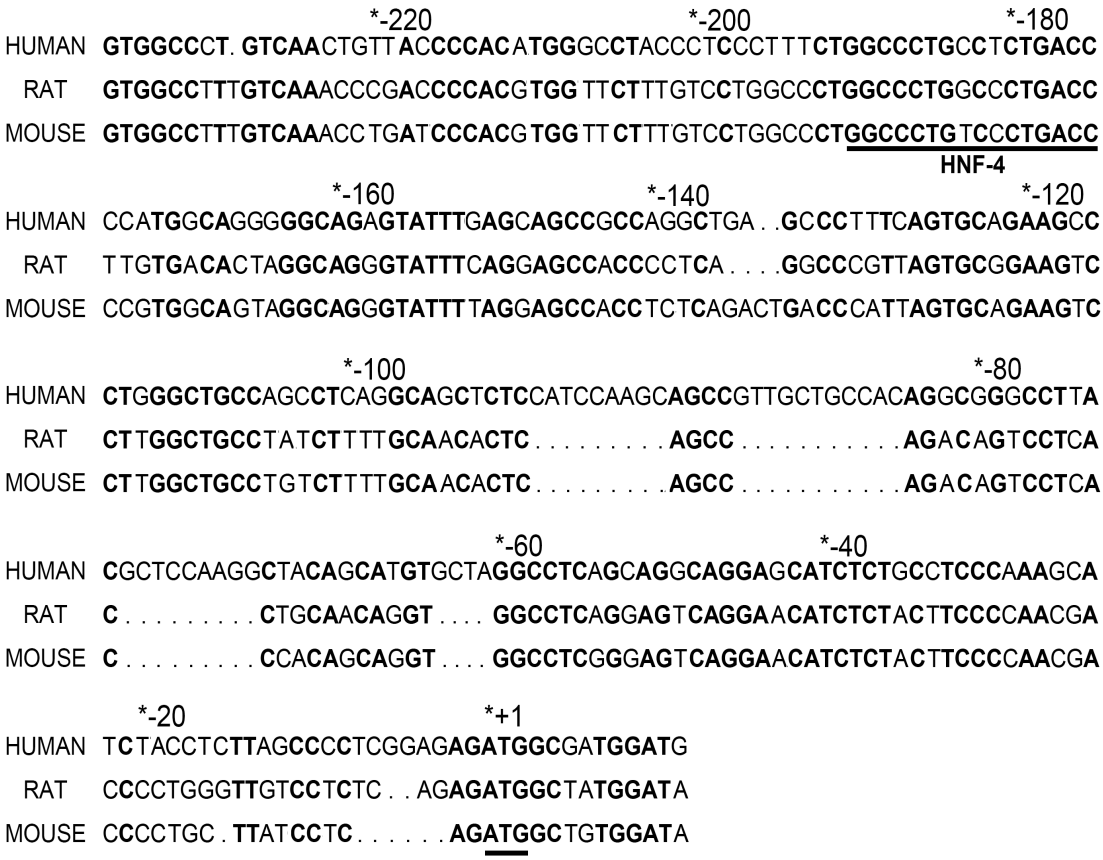

Figure 4.10 Alignment of the human, rat, and mouse downstream promoter DNAs

Bases are numbered with respect to the rat gene. The translation initiation site and putative HNF4 binding site are underlined. Conserved nucleotides are in boldface.

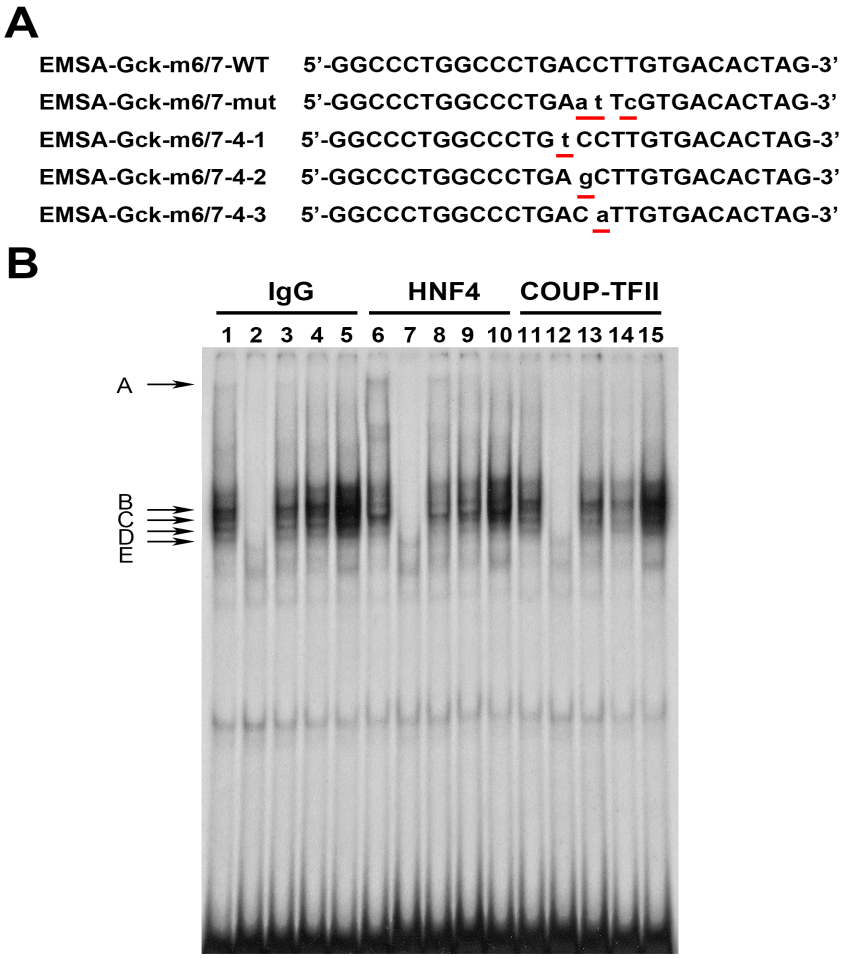

Figure 4.11 Changes in gel shift pattern resulting from mutations in the sequence of hepatic *Gck* -190/-163 oligonucleotide

A, diagram of oligonucleotide probes. Mutated bases are shown in lower-case letters. **B**, 6 μg of nuclear extracts of rat liver were pre-incubated with 1 μg antibodies for 20 m at room temperature as indicated, followed by incubation for 20 m at room temperature with 10^5 cpm of one of the following ^{32}P-labelled probes: *lanes* 1, 6, and 11, EMSA-Gck-m6/7-WT; *lanes* 2, 7, and 12, EMSA-*Gck*-m6/7-mut; *lanes* 3, 8, and 13, EMSA-*Gck*-m6/7-4-1; *lanes* 4, 9, and 14, EMSA-*Gck*-m6/7-4-2; *lanes* 5, 10, and 15, EMSA-*Gck*-m6/7-4-3. The DNA protein binding was analyzed by electrophoresis on 12% (acrylamide/bis-acrylamide ratio: 29:1) native polyacrylamide gels. The gel was exposed to X-ray film for 10 h at room temperature with intensifying screen. A, COUP-TFII, C, HNF4α.

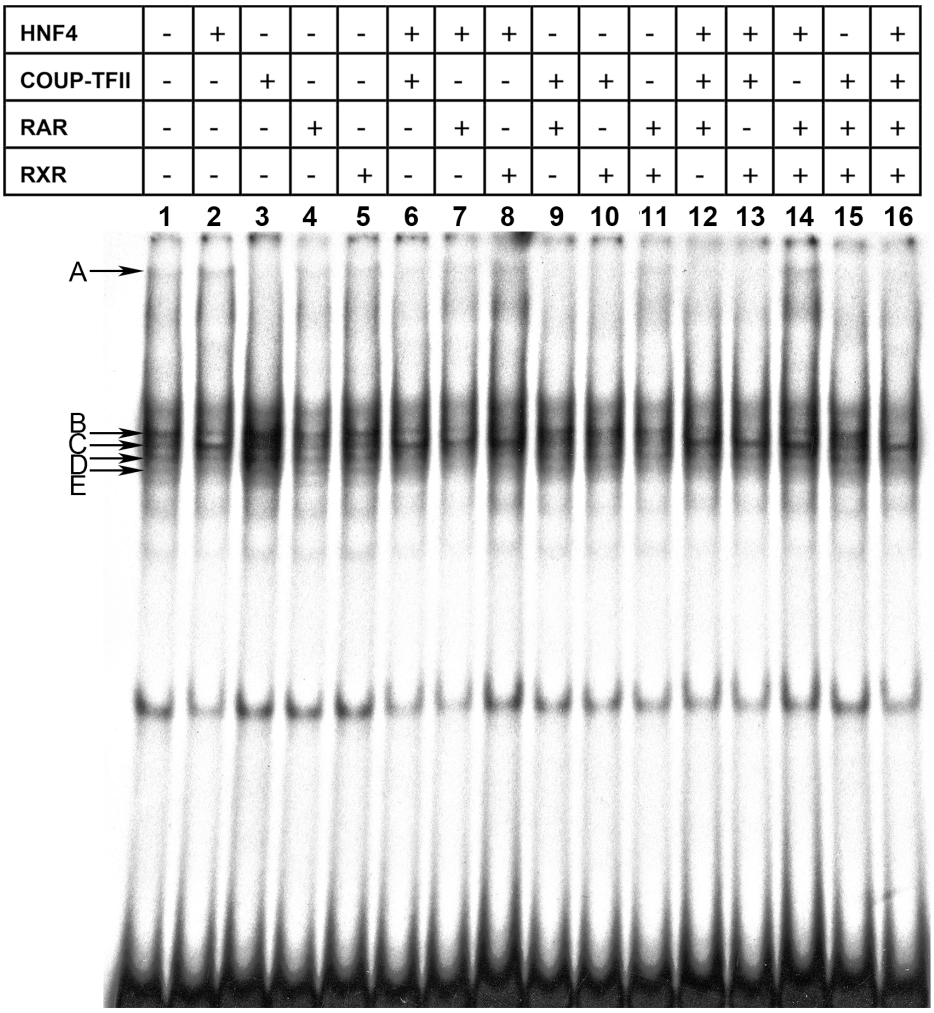

Figure 4.12 Electrophoretic mobility shift assay (EMSA) of binding of HNF4α, COUP-TFII, RAR, and RXR from rat liver nuclear protein extracts to the hepatic *Gck* -190/-163 oligonucleotide

6 µg of nuclear extracts of rat liver were pre-incubated with 4 µg antibodies for 20 m at room temperature as indicated, followed by incubation with 10^5 cpm ^{32}P-labelled EMSA-*Gck*-m6/7-WT oligonucleotides (-190/-163) for 20 minutes at room temperature. The DNA protein binding was analyzed by electrophoresis on 12% (acrylamide/bis-acrylamide ratio: 37.5:1) native polyacrylamide gels. The gel was exposed to X-ray film for 17 h at room temperature with intensifying screen. A, COUP-TFII. B, HNF4α.

833/15 cells over-expressing β-gal, RARα, RXRα, HNF4α, or COUP-TFII, and their binding to EMSA-*Gck*-m6/7-WT was examined by EMSA. INS-1 cells were used here because of the lack of transcription of the downstream *Gck* gene. As shown in Fig. 4.13, recombinant adenovirus-mediated over-expression of these transcriptional factors resulted in increased formation of the indicated complexes, which are not observed in the NEs derived from cells infected by Ad-β-gal.

To determine whether RA affects the formation of the binding complexes or not, the EMSA assay was performed in the absence or presence of increasing concentrations of RA. As shown in Fig. 4.14, the presence of RA at 10 μM increased the formation of all the binding complexes.

We next determined whether the *Gck* promoter in the context of chromatin is bound by these transcriptional factors using chromatin immunoprecipitation (ChIP) assays. Primary rat hepatocytes were infected with Ad-β-gal, Ad-RARα, RXRα, Ad-HNF4α, or Ad-COUP-TFII for 18 h and chromatin was prepared. As shown in Fig. 4.15, these transcription factors were successfully over-expressed. Recombinant adenovirus-mediated over-expression of RARα, RXRα, or COUP-TFII significantly increased their occupancy in *Gck* promoter as compared with Ad-β-gal. However, over-expression of RXRα did not affect the precipitation of *Gck* promoter by RXRα antibody (Fig. 4.16). Despite HL1C hepatoma cells are known not to express *Gck*, RA was able to induce the binding of RARα and HNF4α to the promoter of *Gck* (Fig. 4.17).

Taken together, these data demonstrate that RARE in the hepatic *Gck* promoter is a promiscuous binding site for RAR, RXR, HNF4α, and COUP-TFII and that RA may modulate the binding of nuclear proteins to this site.

Effects of Over-expression of RARα, RXRα, HNF4α, and COUP-TFII on *Gck* and Other Metabolic Genes in Primary Rat Hepatocytes

Given the fact that these transcription factors bind to the RARE, it becomes essential to determine whether any change or shift of the occupancy

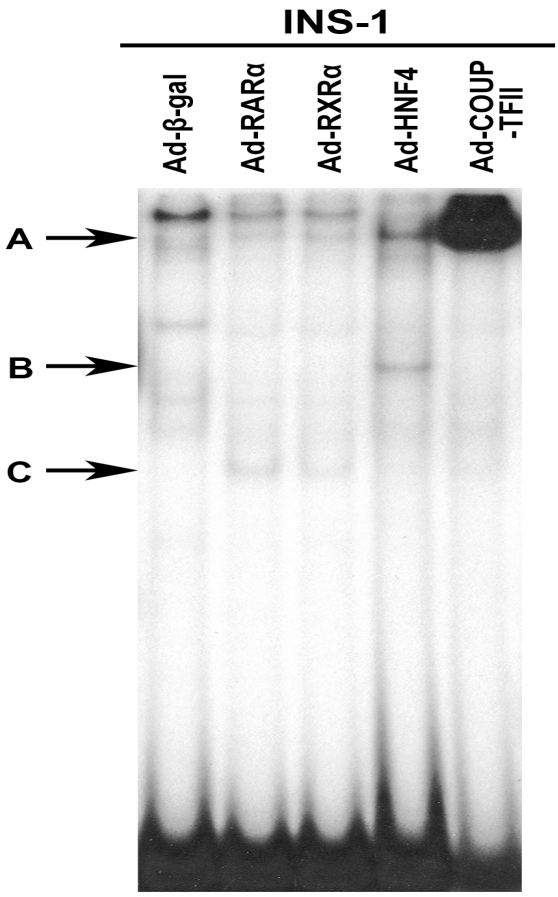

Figure 4.13 Effect of over-expression of RARα, RXRα, HNF4, and COUP-TFII on the EMSA pattern of hepatic *Gck* -190/-163 oligonucleotide in INS-1 cells

2 µg of nuclear extracts prepared from INS-1 833/15 cells infected with adenoviruses Ad-β-gal, Ad-RARα, Ad-RXRα, Ad-HNF4, or Ad-COUP-TFII were incubated with 10^5 cpm ^{32}P-labelled EMSA-Gck-m6/7-WT oligonucleotides (-190/-163) for 20 m at room temperature. The DNA protein binding was analyzed by electrophoresis on 12% (acrylamide/bis-acrylamide ratio: 29:1) native polyacrylamide gels. The gel was exposed to X-ray film for 16 h at room temperature with intensifying screen. A, COUP-TFII, B, HNF4α, C, RARα/RXRα.

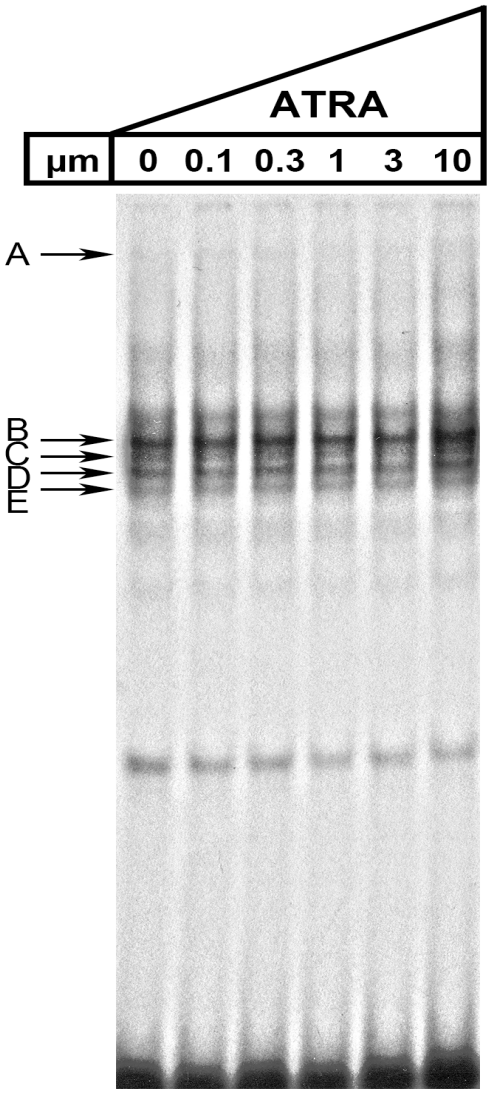

Figure 4.14 Effect of retinoic acid on the EMSA pattern of hepatic *Gck* -190/-163 oligonucleotide

6 µg of nuclear extracts of rat liver were incubated with 10^5 cpm ^{32}P-labelled EMSA-*Gck*-m6/7-WT oligonucleotides (-190/-163) in the presence of the indicated amount of all-*trans* retinoic acid (ATRA) for 20 m at room temperature. The DNA protein binding was analyzed by electrophoresis on 12% (acrylamide/bis-acrylamide ratio: 37.5:1) native polyacrylamide gels. The gel was exposed to X-ray film for 12 h at room temperature with intensifying screen.

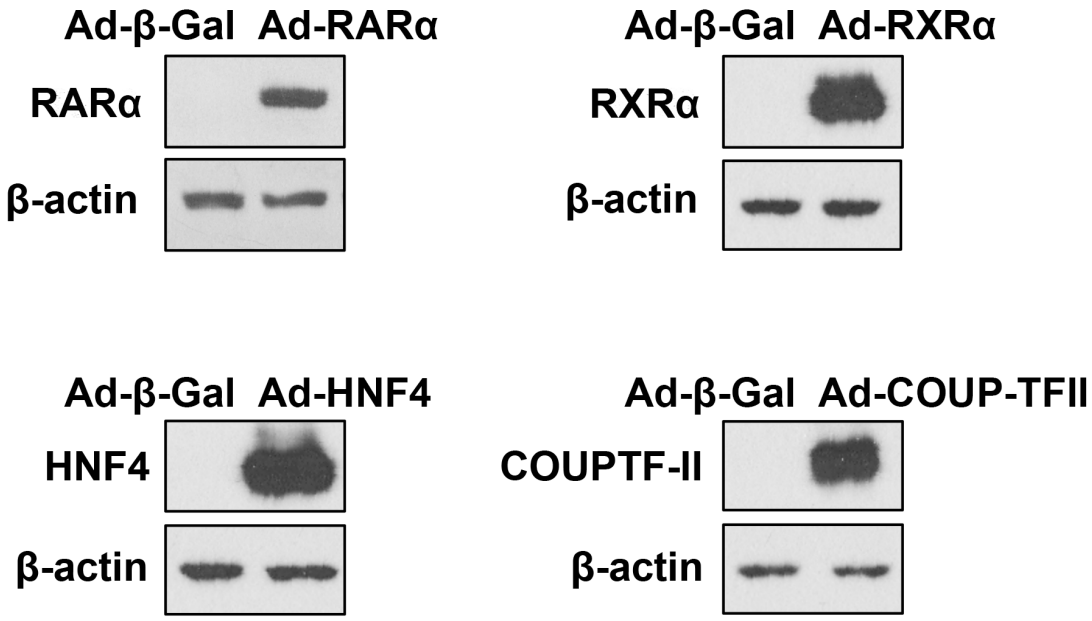

Figure 4.15 The adenovirus-mediated over-expression of nuclear receptors in primary hepatocytes

Primary hepatocytes were infected with Ad-β-gal, Ad-RARα (A), Ad-RXRα (B), Ad-HNF4α (C), or Ad-COUP-TFII (D) at 1,000 PFU/cell for 24 h. Shown are representative Western blots of 20 μg/lane of whole cell lysates showing nuclear receptors (top) and β-actin (bottom) as the loading control.

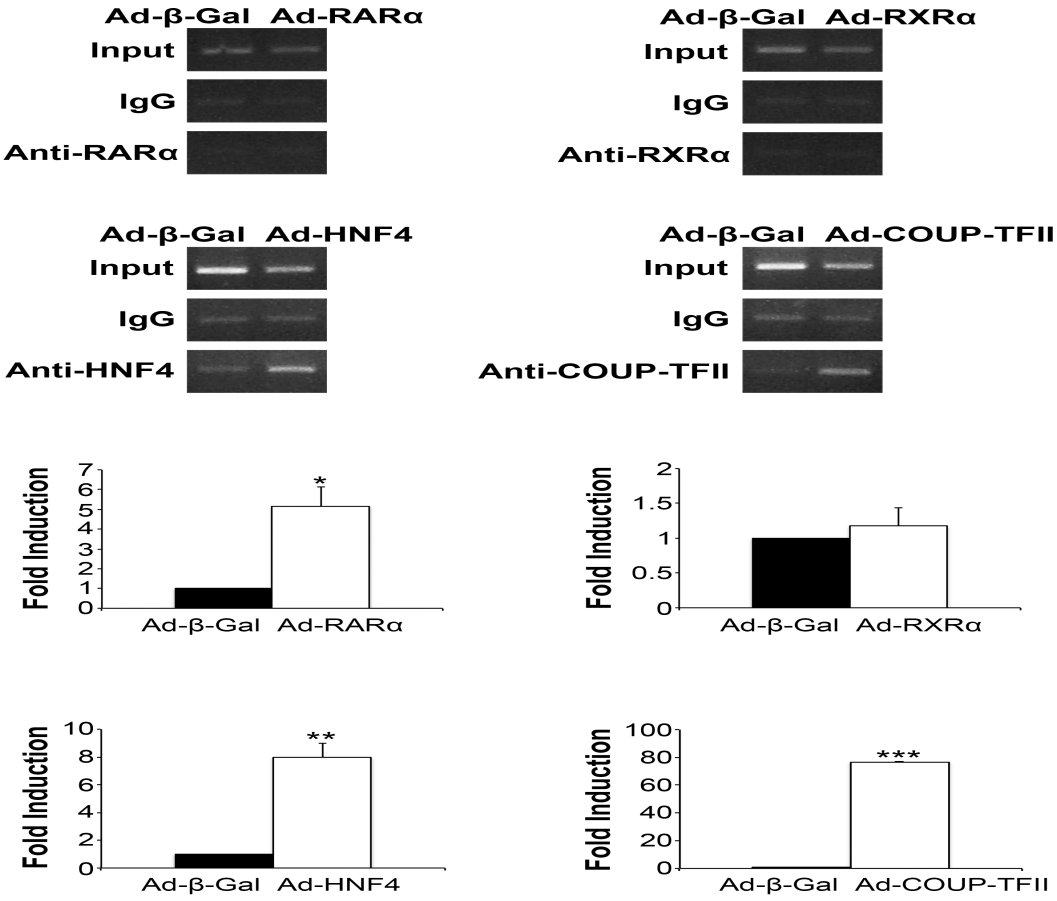

Figure 4.16 Effect of recombinant adenoviruses on immunoprecipitation of the *Gck* promoter-containing chromatin from primary hepatocytes with RARα, RXRα, HNF4α, and COUP-TFII antibodies

Primary hepatocytes were infected with Ad-β-gal, Ad-RARα, Ad-RXRα, Ad-HNF4α, or Ad-COUP-TFII at 1,000 PFU/cell for 18 h and crosslinked with formaldehyde and immunoprecipitated with the antibody against RARα, RXRα, HNF4, and COUP-TFII or with control IgG. Sonicated genomic DNA from the nuclei treated with antibody or IgG and the nuclei not treated with antibody (input) were isolated and amplified by PCR for a 250 bp *Gck* promoter region using *Gck*-specific primers. PCR products were resolved on agarose gels containing ethidium bromide. * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$ for comparing indicated adenovirus with β-gal.

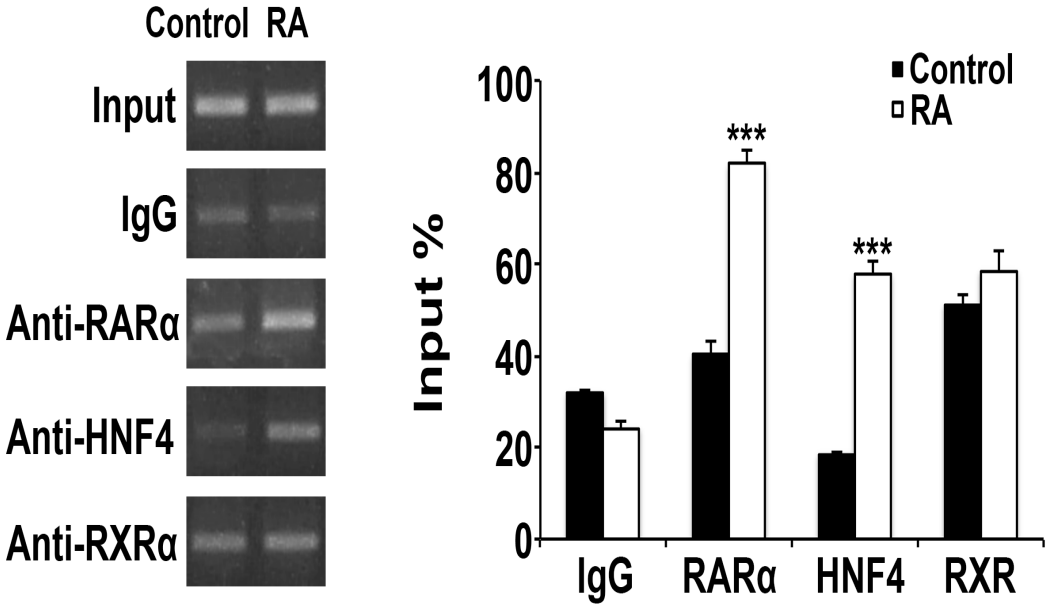

Figure 4.17 Immunoprecipitation of the *Gck* promoter-containing chromatin from HL1C hepatoma cells with RARα, RXRα, and HNF4α antibodies

HL1C cells treated without or with 1 μM RA for 6 h were crosslinked with formaldehyde and immunoprecipitated with the antibody against RARα, RXRα, and HNF4α, or with control IgG. Sonicated genomic DNA from the nuclei treated with antibody or IgG and the nuclei not treated with antibody (input) were isolated and amplified by PCR for a 250 bp *Gck* promoter region using *Gck*-specific primers. PCR products were resolved on agarose gels containing ethidium bromide. *** $p < 0.001$ for comparing with control.

of one of these transcription factors will influence *Gck* expression in the presence or absence of RA an insulin. As shown in Fig. 4.18, in β-gal adenovirus control group, RA and insulin increased *Gck* mRNA expression level by 5.6- and 53.7-fold, respectively. RA + insulin synergistically induced its mRNA level by 397-fold. Recombinant adenovirus-mediated over-expression of RARα, RXRα, or HNF4α had little effect on the basal transcription of *Gck*. However, over-expression of COUP-TFII significantly reduced basal transcription of *Gck* by 56%. Over-expression of RARα potentiated RA-induced *Gck* transcription by 44.6-fold, an effect not observed in RXRα over-expression group. In contrast, over-expression of HNF4α attenuated RA-induced *Gck* transcription by 71.0% and over-expression of COUP-TFII completely abolished the induction. Whereas over-expression of RXRα did not affect insulin-induced *Gck* transcription, over-expression of RARα, HNF4α, and COUP-TFII suppressed insulin-induced *Gck* transcription by 85.9%, 96.0%, and 90.0%, respectively. Synergistic induction of *Gck* transcription by RA and insulin was not affected by over-expression of RXRα, increased by 5.2-fold by over-expression of RARα, and almost completely abolished by over-expression of HNF4α and COUP-TFII (99.3% and 96.8%, respectively).

To confirm that insulin and RA treatment worked, we measured the expression of *Pck1*, which is regulated by both RA and insulin. As shown in Fig. 4.19, over-expression of RXRα had little effect on basal expression level of *Pck1* mRNA. However, over-expression of RARα and COUP-TFII suppressed the basal expression of *Pck1* mRNA by 85% and 81%, respectively. RA induced the mRNA level of *Pck1* in β-galactosidase adenovirus control group by 2.7-fold, which, without being affected by over-expression of RARα or RXRα, was attenuated by 83.5% in the presence of over-expressed COUP-TFII. As expected, insulin dramatically suppressed the expression level of *Pck1* mRNA in control group and none of the adenoviruses had any further effect. RA and insulin together restored the transcription of *Pck1* to basal level in control group. However, this effect was antagonized by

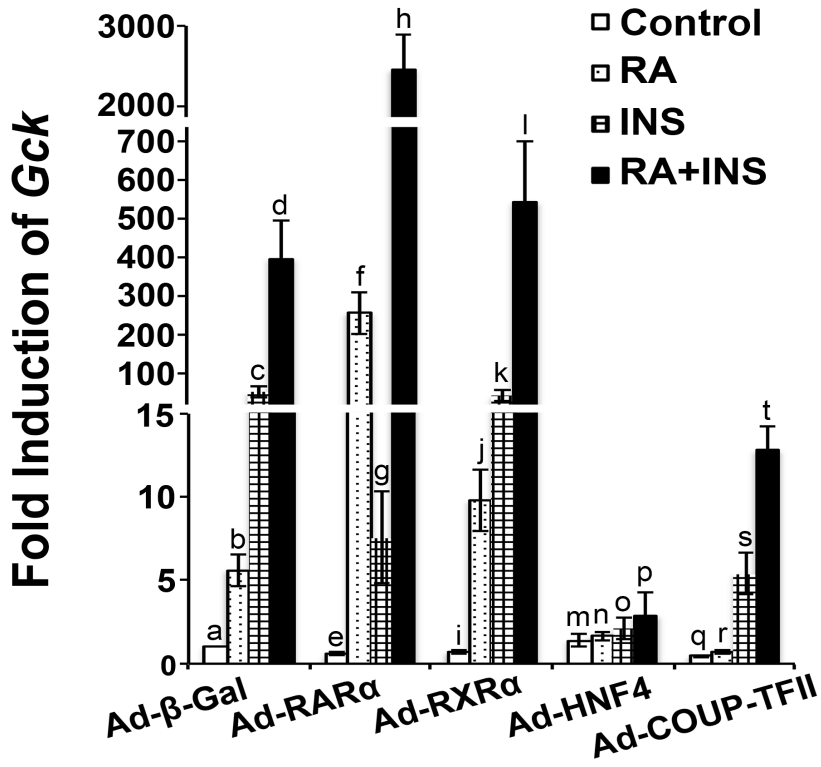

Figure 4.18 Effect of over-expression of RARα, RXRα, HNF4α, and COUP-TFII on *Gck* mRNA expression in primary hepatocytes

Primary hepatocytes were infected with Ad-β-gal, Ad-RARα, Ad-RXRα, Ad-HNF4α, or Ad-COUP-TFII at 1,000 PFU/cell for 18 h, followed by treatment without or with 5 μM RA in the absence or presence of 1 nM insulin for 6 h, after which the cells were harvested and total RNA was extracted and subjected to real-time PCR analysis. The mRNA level of *Gck* in control hepatocytes infected with Ad-β-gal and treated with vehicle control was arbitrarily assigned a value of 1. Each value represents the mean ± SD of four independent treatments ($a < b < c < d$, $e < g < f < h$, $i < j < k < l$, $q < r < s < t$, $a > q$, $r < n < b < j < f$, $o < g/s < c/k$, $p < t < d/l < h$ for comparing C, RA, INS, and RA + INS in the presence of indicated adenoviruses, and for comparing each treatment in the presence of different adenoviruses using one way ANOVA; all $p < 0.05$).

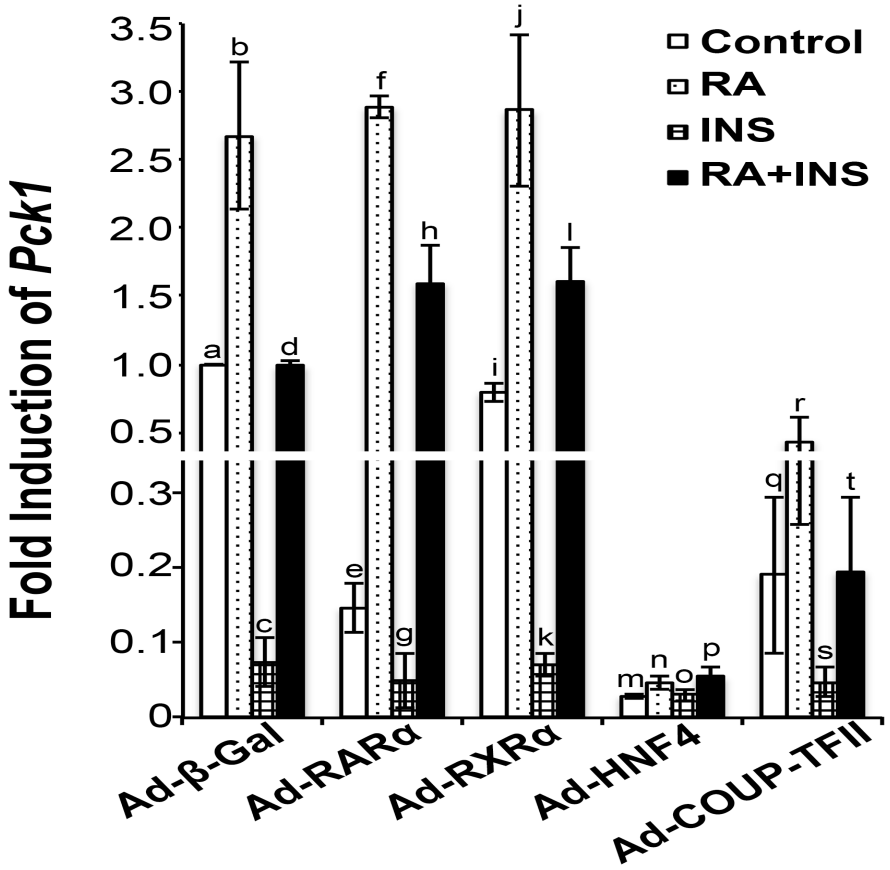

Figure 4.19 Effect of over-expression of RARα, RXRα, HNF4α, and COUP-TFII on *Pck1* mRNA expression in primary hepatocytes

Primary hepatocytes were infected with Ad-β-gal, Ad-RARα, Ad-RXRα, Ad-HNF4α, or Ad-COUP-TFII at 1,000 PFU/cell for 18 h, followed by treatment without or with 5 μM RA in the absence or presence of 1 nM insulin for 6 h, after which the cells were harvested and total RNA was extracted and subjected to real-time PCR analysis. The mRNA level of *Pck1* in control hepatocytes infected with Ad-β-gal and treated with vehicle control was arbitrarily assigned a value of 1. Each value represents the mean ± SD of four independent treatments (c < a/d < b, e/g < f/h, k < i < l < j, m < n, m/o < p, s < r, m < e < a/i, m < q < a, n < r < b/f/j, p/t < d/h/l for comparing C, RA, INS, and RA + INS in the presence of indicated adenoviruses, and for comparing each treatment in the presence of different adenoviruses using one way ANOVA; all $p < 0.05$).

over-expression of COUP-TFII. The mRNA expression of *Pck1* was eliminated in cells over-expressing of HNF4α under all conditions.

To determine the responses of an insulin-suppressed gene that is not affected by RA, the expression levels of *Igfbp1* mRNA were measured in the same samples. As shown in Fig. 4.20 in β-galactosidase adenovirus control group, RA had no effect on *Igfbp1* transcription, whereas insulin suppressed its mRNA expression by about 60% independent of the presence of RA. Neither over-expression of RARα nor RXRα had any effect on the basal transcription of *Igfbp1*. However, over-expression of COUP-TFII reduced the basal mRNA level of *Igfbp1* by 58%. In the presence of RA, over-expression of RARα, RXRα, and COUP-TFII reduced the transcription of *Igfbp1* by 50%, 39%, and 55%, respectively. Only over-expression of HNF4α resulted in change of insulin-suppressed *Igfbp1* expression. In the presence of both RA and insulin, over-expression of RXRα and COUP-TFII did not affect *Igfbp1* transcription, which was almost eliminated by over-expression of RARα. The transcription of *Igfbp1* was eliminated by over-expression of HNF4α under all conditions.

To determine the effects of over-experssion of these NRs on another gene which is induced by both RA and insulin, we measured the expression levels of *Srebp-1c* in the same cells. As shown in Fig. 4.21, in β-galactosidase adenovirus control group, RA and insulin alone induced *Srebp-1c* mRNA expression by 2- and 4-fold, respectively. RA and insulin synergistically increased *Srebp-1c* mRNA level up to 32.6-fold. Basal mRNA level of *Srebp-1c*, without being affected by over-expression of RARα and RXRα, was increased by 2.8-fold and decreased by 60% by over-expression of HNF4α and COUP-TFII, respectively. Over-expression of RARα, RXRα, and HNF4α augmented RA-induced *Srebp-1c* transcription by 1.4-, 2.2-, and 0.8-fold, respectively. Insulin-induced *Srebp-1c* transcription was abolished by over-expression of RARα and COUP-TFII, without being affected by over-expression of RXRα and HNF4α. Synergistic inducion of *Srebp-1c* transcription was not affected by over-expression of RARα, potentiated by

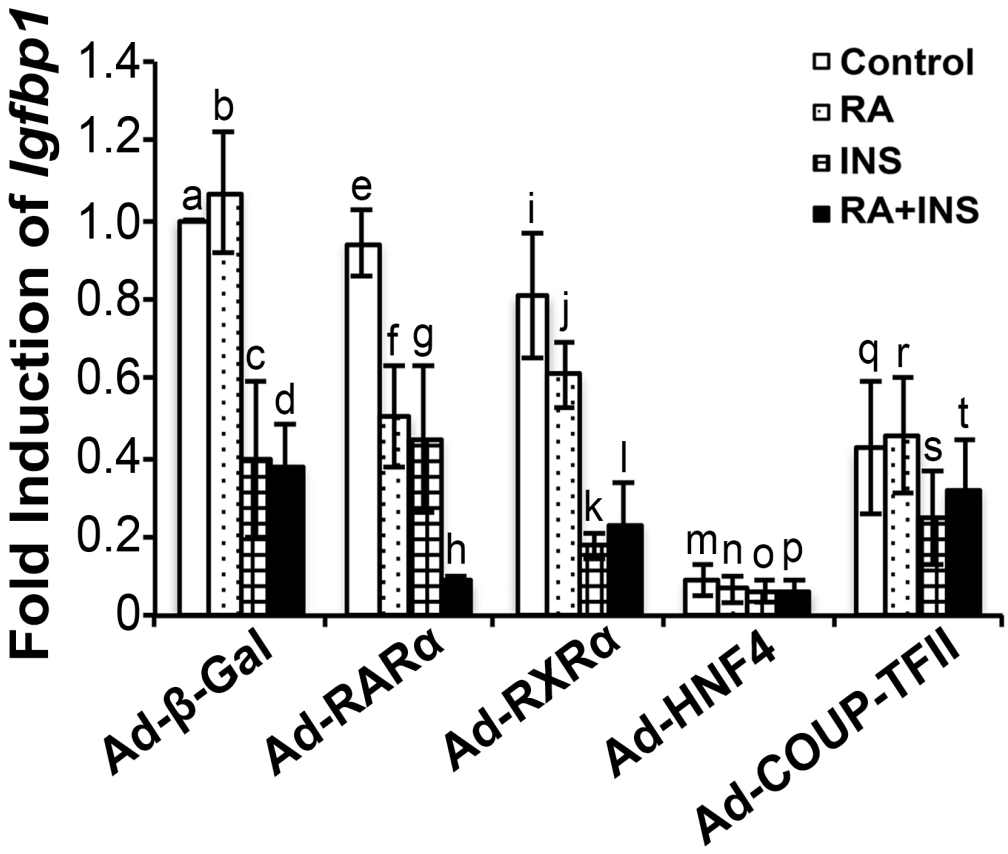

Figure 4.20 Effect of over-expression of RARα, RXRα, HNF4α, and COUP-TFII on *Igfbp1* mRNA expression in primary hepatocytes

Primary hepatocytes were infected with Ad-β-gal, Ad-RARα, Ad-RXRα, Ad-HNF4α, or Ad-COUP-TFII at 1,000 PFU/cell for 18 h, followed by treatment without or with 5 μM RA in the absence or presence of 1 nM insulin for 6 h, after which the cells were harvested and total RNA was extracted and subjected to real-time PCR analysis. The mRNA level of *Igfbp1* in control hepatocytes infected with Ad-β-gal and treated with vehicle control was arbitrarily assigned a value of 1. Each value represents the mean ± SD of four independent treatments (c/d < a/b, h < f/g < e, k/l < i/j, m < q < a/e/i, n < f/j/r < b, o < c/g/k/s, h/p < d/l/t for comparing C, RA, INS, and RA + INS in the presence of indicated adenoviruses, and for comparing each treatment in the presence of different adenoviruses using one way ANOVA; all $p < 0.05$).

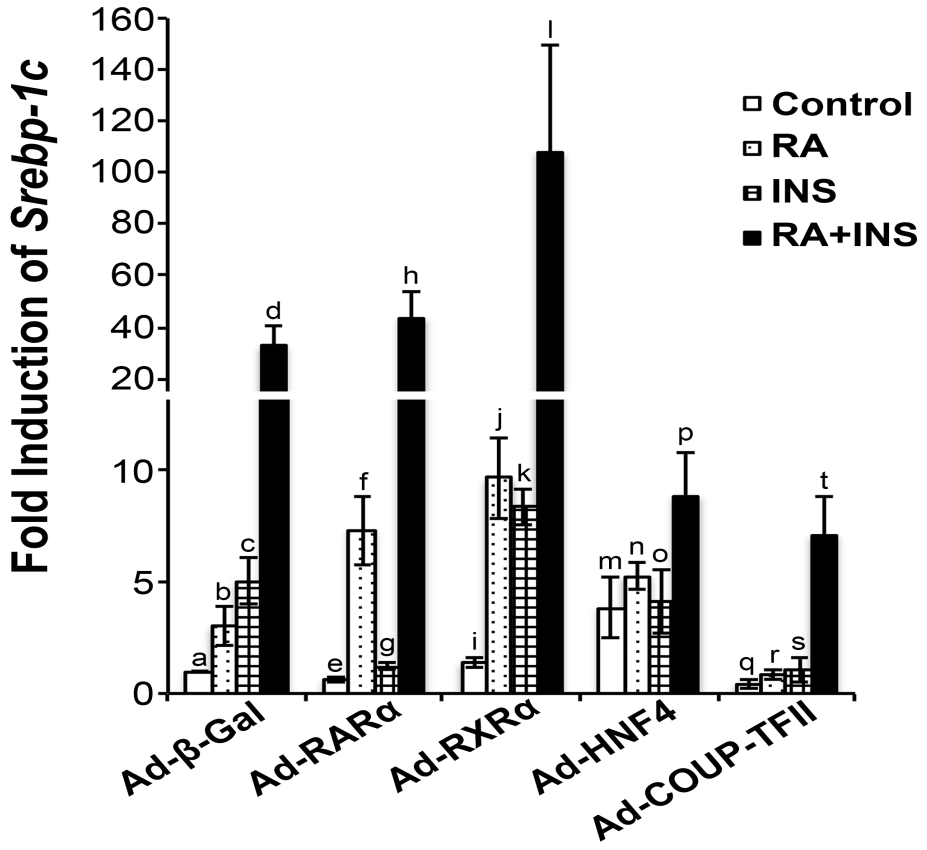

Figure 4.21 Effect of over-expression of RARα, RXRα, HNF4α, and COUP-TFII on *Srebp-1c* mRNA expression in primary hepatocytes

Primary hepatocytes were infected with Ad-β-gal, Ad-RARα, Ad-RXRα, Ad-HNF4α, or Ad-COUP-TFII at 1,000 PFU/cell for 18 h, followed by treatment without or with 5 μM RA in the absence or presence of 1 nM insulin for 6 h, after which the cells were harvested and total RNA was extracted and subjected to real-time PCR analysis. The mRNA level of *Srebp-1c* in control hepatocytes infected with Ad-β-gal and treated with vehicle control was arbitrarily assigned a value of 1. Each value represents the mean ± SD of four independent treatments ($a < b/c < d$, $e < g < f < h$, $i < j/k < l$, $m/n < p$, $q/r/s < t$, $q < a < m$, $e/q < i < m$, $r < b < f$, $r < b < n < j$, $g/s < c$, $g/s < o < k$, $p/t < d/h < l$ for comparing C, RA, INS, and RA + INS in the presence of indicated adenoviruses, and for comparing each treatment in the presence of different adenoviruses using one way ANOVA; all $p < 0.05$).

2.3-fold with over-expression of RXRα, abolished by over-expression of HNF4α and COUP-TFII.

4.4 Discussion

As an energy source, glucose homeostasis is delicately regulated. The expression of two GK isoforms in different cells serves the purpose to control the transcription initiation by distinct sets of *cis*-acting regulatory elements in different cellular environment in response to the same nutrient and hormonal stimuli. The regulation of the hepatic (downstream) *Gck* promoter is more complex and less understood than that of the β cell promoter. It has been shown that the β cell *Gck* promoter contains a putative RARE (-196/-154). However, no classical RARE consensus appears to be present on the hepatic *Gck* promoter [337]. In the present study, we identify a RARE in the hepatic *Gck* promoter and demonstrate that this RARE is a convergent site for NRs RARα, RXRα, HNF4α, and COUP-TFII to regulate hepatic *Gck* expression.

Hepatic GK is regulated by nutritional and hormonal stimuli, of which insulin is the most potent. During rat development, GK expresssion first appears in the liver 2 weeks after birth and its activity rapidly increases after weaning in response to feeding of a high-carbohydrate diet due to the increase of plasma insulin and the decrease of plasma glucagon concentrations [338, 339]. In contrast, GK mRNA and protein disappear in the liver of insulin-deficient rats and are restored after insulin treatment [340]. It was reported that insulin-induced GK activity was mainly resulted from alterations in the mRNA level [341]. In a previous study [12], we demonstrated that insulin was able to synergize with RA to induce hepatic *Gck* mRNA expression in 3 h, which is consistent with a recent report showing that insulin triggered a 15- to 30-fold increase in hepatic *Gck* mRNA in 3 h independent of the presence of glucose [87]. The rapid effect of insulin on hepatic *Gck* mRNA expression indicates that insulin directly stimulates hepatic *Gck* transcription, supporting the previous observations [340]. However, the reporter gene constructs used in this study is unable to mediate insulin-stimulated *Gck*

transcription (Fig. 4.1). This phenomenon supported by another group's work which could not identify the insulin responsive element (IRE) in primary hepatocytes using a reporter gene construct with a 5.5 kb hepatic *Gck* promoter fragment [341].

Introduction of expression plasmids containing RARα and RXRγ improved the fold induction of promoter activity by RA, but not insulin, allowing us to determine the RARE in hepatic *Gck* promoter. A scan of the *Gck* locus for DNase-I hypersensitive sites revealed that two hypersensitive proximal sites, both of which were located within the 1 kb of promoter, might be involved in the regulation of *Gck* by insulin [342]. However, the insulin-response sequence like that is located near the hypersensitive sites was not able to confer a positive effect of insulin in transfection experiments [342]. These observations suggest that transient transfection may not be an ideal technique to identify the IRE on hepatic *Gck* promoter and new strategies are necessary in order to gain an insight into the hormonal regulation of the transcription of hepatic *Gck*.

Recently, Kim *et al.* [121] reported that LXRα can increase hepatic *Gck* expression by directly binding to a functional LXR response element (LXRE) on the *Gck* promtor, which overlaps the RARE identified in the present study. In the former study, the authors performed transient transfection in Alexander human hepatoma cells and did not observe any induction of the *Gck* promoter (~ 1.5 kb) activity by T1317 and/ or 9-*cis* RA in the absence of expression plasmids containing LXRα and/ or RXRα. Similarly, our *Gck* promoter construct (~ 1kb) did not respond to treatment of all-*trans* RA in HL1C hepatoma cells (data not shown) or in INS-1 833/15 cells (Fig. 4.3) in the absence of expression plasmids containing RARα and/ or RXRγ. However, all-*trans* RA was able to significantly induce the 1k *Gck* promoter acitivity in primary rat hepatocytes as shown in Fig. 4.1 and Fig. 4.2. This induction was further augmented by co-transfection of RARα, RXRα, and/ or RXRγ expression vectors. The differential response of hepatic *Gck* promoter to the abovementioned ligands in different cells may be attributed to the different

expression levels of NRs that mediating the promoter activation. In addition, Kim *et al.* could not rule out the possibility that heterodimer RAR/RXR might also play a role in mediating the induction of *Gck* promoter activity in the presence of 9-*cis*, which is also capable of activating RAR.

It has been suggested that whether TTNPB (a specific agonist of RAR) mediated-RAR activation in primary hepatocytes attenuates or potentiates insulin-regulated gene expression depends on the promoter context, since TTNPB differentially regulates insulin-induce mRNA expression of *Gck* and *Srebp-1c* [12]. However, the induction patterns of mRNA expression of *Gck* and *Srebp-1c* by over-expression of RARα were similar in the present study. We found that over-expression of RARα attenuated the insulin-induced *Gck* and *Srebp-1c* mRNA expression in the absence of RA. On the other hand, the synergistic effect between RA and insulin on hepatic *Gck* and *Srebp-1c* mRNA expression was further boosted by RARα over-expression (Fig. 4.18 and Fig. 4.21). It is possible that activated RARα by RA or by TTNPB function differentially in the same promoter context (*Srebp-1c* promoter). Another possibility is that high levels of unliganded RARα can interfere with insulin signaling pathway. The presence of RA results in a repartition of liganded RARα from signaling cascade to the RARE on target gene promoters. Further studies will be necessary to reveal the mechanism.

In the liver, GK expression shows a slight predominance in the less aerobic, perivenous zone [343]. It has been shown that HNF4α may play a role in the O_2-mediated zonated *Gck* expression in rat liver [130]. The expression of HNF4α in rat hepatocytes cultured under perivenous pO_2 (8% O_2) is significantly higher than that under periportal pO_2 (16% O_2). Transfection of the HNF4α expression vector into hepatocytes increases GK mRNA, protein, and enzyme activity under both culturing conditions [130]. In the present study, however, recombinant adenovirus-mediated over-expression of HNF4α did not result in significant increase in *Gck* mRNA expression in rat hepatocytes cultured in a more aerobic atmosphere (~20% O_2) (Fig. 4.18). Whether the discrepancy is due to different culturing

conditions merits further investigation. Althought it is well established that GK is regulated by nutritional (e.g. RA) and hormonal (e.g. insulin) stimuli, the role of HNF4α in this process has not been well addressed. We demonstrate that RA- and insulin-induced *Gck* mRNA expression is markedly suppressed by over-expression of HNF4α, indicating that HNF4α may function as a negative regulator for the hepatic *Gck* expression. The inhibition may be caused by the direct binding of HNF4α to the RARE as ChIP assay results shown in Fig. 4.16 demonstrated the increased occupancy at RARE after its overexpression.

COUP-TFII plays an important role in regulating metabolism. It has been suggested that COUP-TFII can modulate RA signaling pathway in embryogenesis (see Chapter 2), but little information is available about the effect of COUP-TFII on RA-induced expression of genes involved in glucose and lipid metabolism. We show here that recombinant adenovirus-mediated over-expression of COUP-TFII suppresses expression of RARs and RXRs in hepatocytes (Fig. 4.22-4.25), which is accompanied by markedly suppression of basal and activated mRNA expression of *Gck, Srebp-1c*, *Pck1*, and *G6pase*. The COUP-TFII effects on these genes' expression can be attributed to several reasons. First, COUP-TFII might modulate the expression of RARs and RXRs and in turn, affects their expression. Second, COUP-TFII occupied the RARE upon over-expression and functioned as a suppressor of these genes' expression in hepatocytes. Last, COUP-TFII could affect these gene's expression indirectly via the control of HNF4α expression. Further studies are needed to determine which one is relevant.

The interplay between NRs RARα, RXRα, HNF4α, and COUP-TFII has been observed in different cell types and promoter contexts. For example, RXR, HNF4, and COUP-TFII interact to modulate transcription of the cholesterol 7α-hydroxylase gene [344, 345]. A common binding site for RAR, RXR, HNF4, and COUP-TFII was also identified on the mouse cellular retinol-binding protein II gene promoter [197]. In the present study, we demonstrate that RARα, RXRα, HNF4α, and COUP-TFII bind to the RARE on the proximal

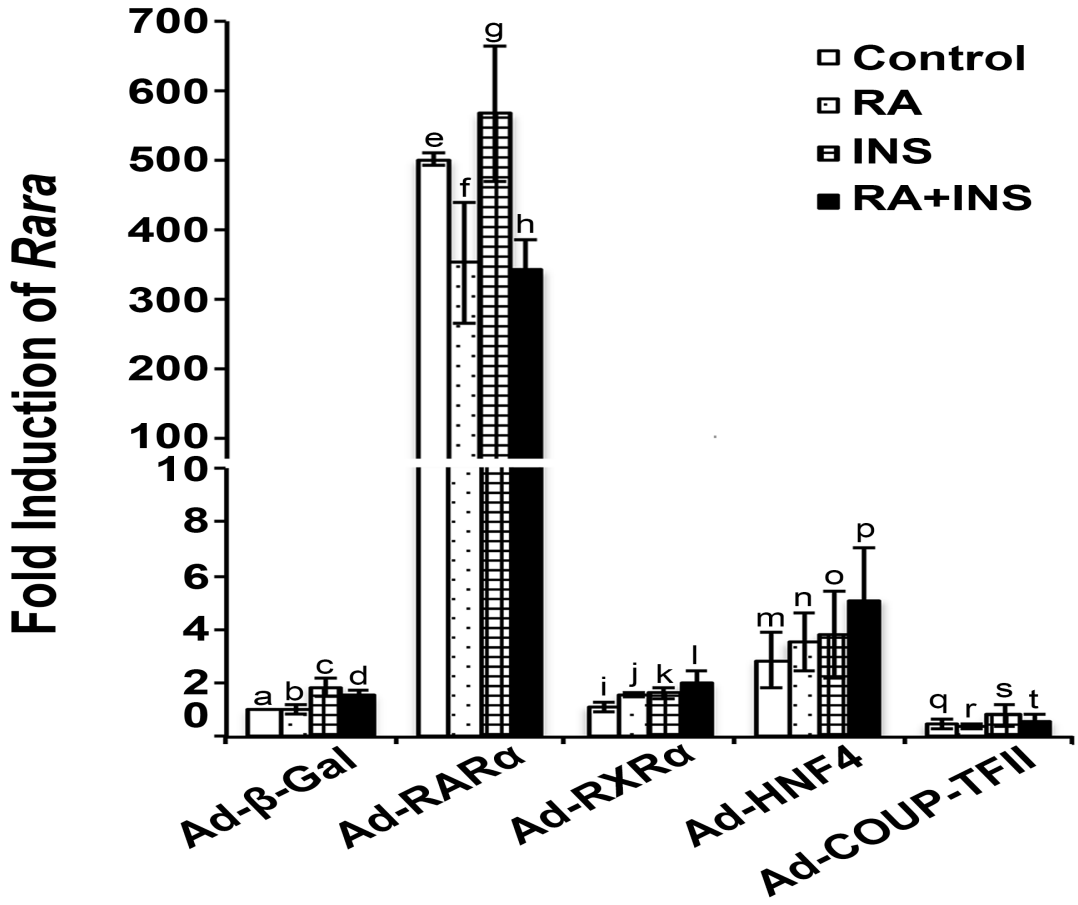

Figure 4.22 Effect of over-expression of RARα, RXRα, HNF4α, and COUP-TFII on mRNA expression of *Rara* in primary hepatocytes

Primary hepatocytes were infected with Ad-β-gal, Ad-RARα, Ad-RXRα, Ad-HNF4α, or Ad-COUP-TFII at 1,000 PFU/cell for 18 h, followed by treatment without or with 5 μM RA in the absence or presence of 1 nM insulin for 6 h, after which the cells were harvested and total RNA was extracted and subjected to real-time PCR analysis. The mRNA level of *Rara* in control hepatocytes infected with Ad-β-gal and treated with vehicle control was arbitrarily assigned a value of 1. Each value represents the mean ± SD of four independent treatments (f/h < e/g, m< p, a/i/q < m < e, b/j/r < n < f, c/k/s < o < g, d/l/t < p < h for comparing C, RA, INS, and RA + INS in the presence of indicated adenoviruses, and for comparing each treatment in the presence of different adenoviruses using one way ANOVA; all $p < 0.05$)

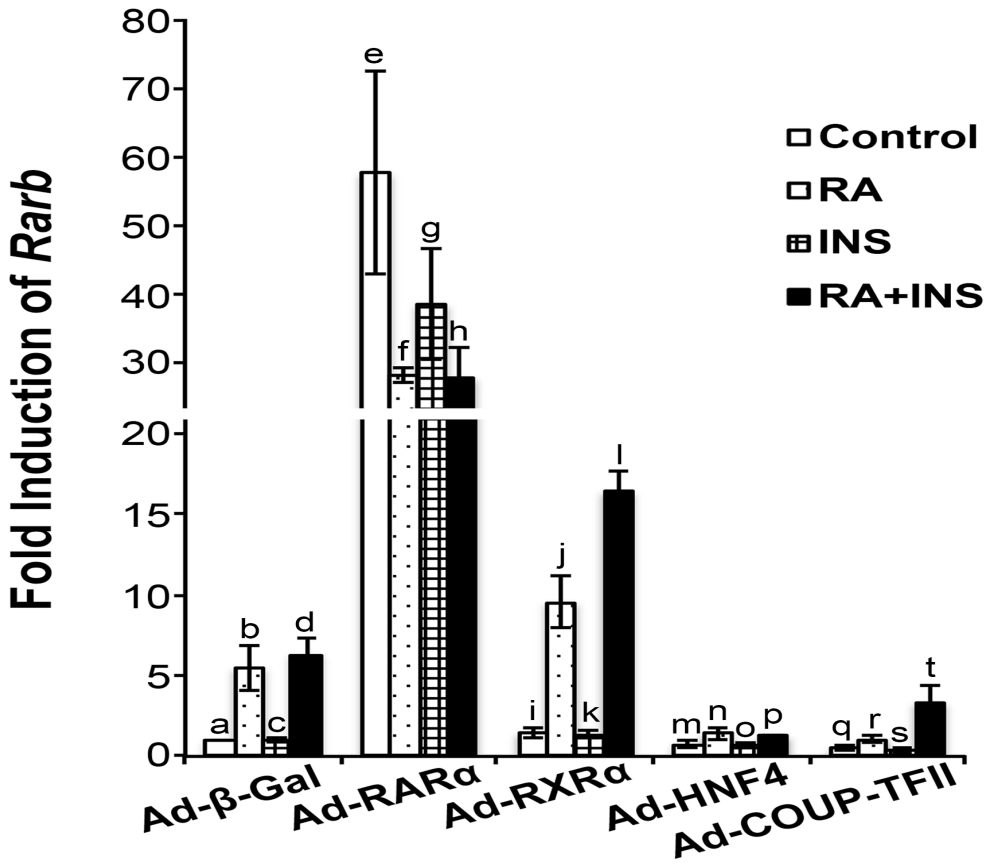

Figure 4.23 Effect of over-expression of RARα, RXRα, HNF4α, and COUP-TFII on mRNA expression of *Rarb* in primary hepatocytes

Primary hepatocytes were infected with Ad-β-gal, Ad-RARα, Ad-RXRα, Ad-HNF4α, or Ad-COUP-TFII at 1,000 PFU/cell for 18 h, followed by treatment without or with 5 μM RA in the absence or presence of 1 nM insulin for 6 h, after which the cells were harvested and total RNA was extracted and subjected to real-time PCR analysis. The mRNA level of *Rarb* in control hepatocytes infected with Ad-β-gal and treated with vehicle control was arbitrarily assigned a value of 1. Each value represents the mean ± SD of four independent treatments (a/c < b/d, f/h < e, f/h < g, i/k < j < l, q/s < t, r < t, a/i/m/q < e, n/r < b < j < f, c/k/o/s < g, p < t < d < l < h for comparing C, RA, INS, and RA + INS in the presence of indicated adenoviruses, and for comparing each treatment in the presence of different adenoviruses using one way ANOVA; all $p < 0.05$).

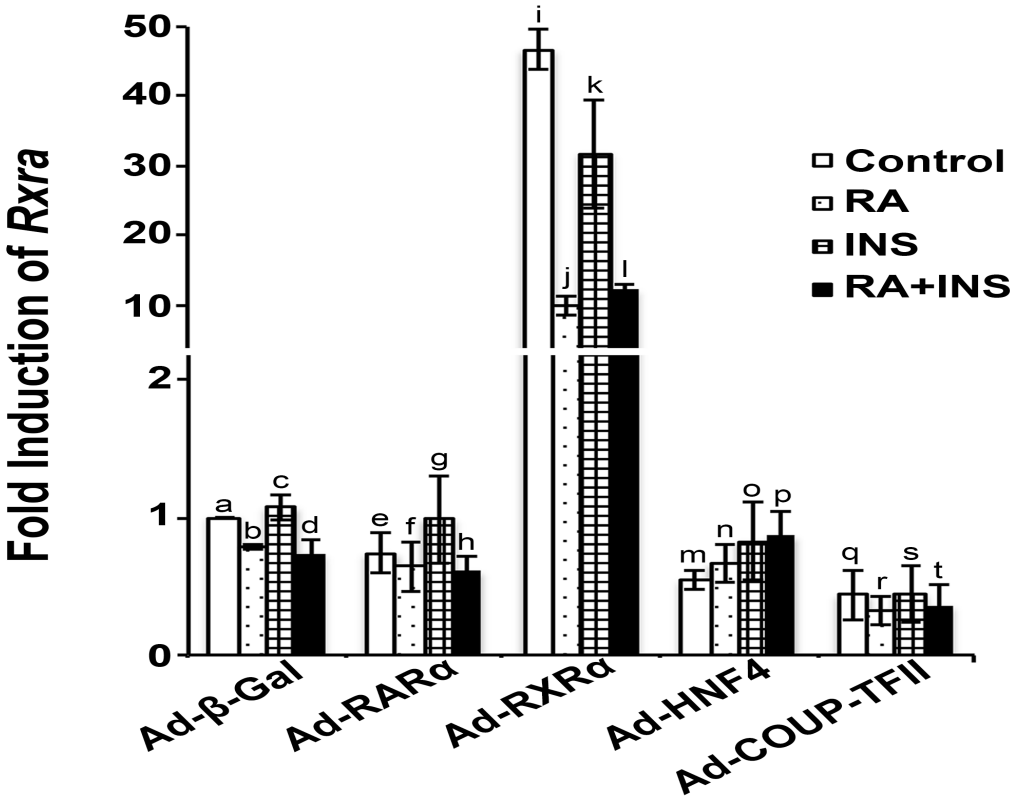

Figure 4.24 Effect of over-expression of RARα, RXRα, HNF4α, and COUP-TFII on mRNA expression of *Rxra* in primary hepatocytes

Primary hepatocytes were infected with Ad-β-gal, Ad-RARα, Ad-RXRα, Ad-HNF4α, or Ad-COUP-TFII at 1,000 PFU/cell for 18 h, followed by treatment without or with 5 μM RA in the absence or presence of 1 nM insulin for 6 h, after which the cells were harvested and total RNA was extracted and subjected to real-time PCR analysis. The mRNA level of *Rxra* in control hepatocytes infected with Ad-β-gal and treated with vehicle control was arbitrarily assigned a value of 1. Each value represents the mean ± SD of four independent treatments (j/l < k < i, a/e/m/q < i, b/f/n/r < j, c/g/o/s < k, d/h/p/t < l for comparing C, RA, INS, and RA + INS in the presence of indicated adenoviruses, and for comparing each treatment in the presence of different adenoviruses using one way ANOVA; all $p < 0.05$).

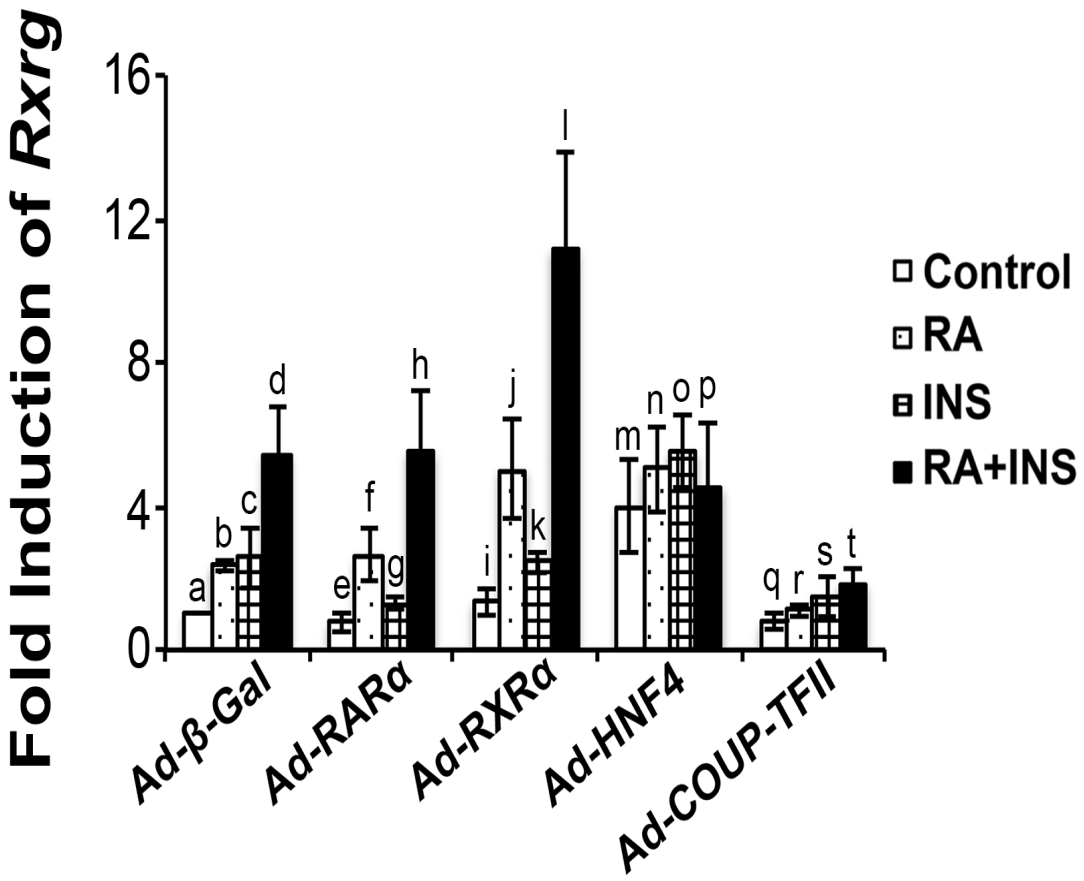

Figure 4.25 Effect of over-expression of RARα, RXRα, HNF4α, and COUP-TFII on mRNA expression of *Rxrg* in primary hepatocytes

Primary hepatocytes were infected with Ad-β-gal, Ad-RARα, Ad-RXRα, Ad-HNF4α, or Ad-COUP-TFII at 1,000 PFU/cell for 18 h, followed by treatment without or with 5 μM RA in the absence or presence of 1 nM insulin for 6 h, after which the cells were harvested and total RNA was extracted and subjected to real-time PCR analysis. The mRNA level of *Rarg* in control hepatocytes infected with Ad-β-gal and treated with vehicle control was arbitrarily assigned a value of 1. Each value represents the mean ± SD of four independent treatments (a < b/c < d, e/g < f < h, i < k < j < l, a/e/i/q < m, r < b/f < j/n, g/s < c/k < o, t < d/h/p < l for comparing C, RA, INS, and RA + INS in the presence of indicated adenoviruses, and for comparing each treatment in the presence of different adenoviruses using one way ANOVA; all $p < 0.05$).

hepatic *Gck* promoter and regulate the transcription of *Gck*, with the former two functioning as positive regulators and the latter two functioning as negative regulators. RAR and RXR isoforms exhibit different expression patterns. *Rara*, *Rxra*, and *Rxrb* are widely expressed, which resembles the pattern of expression of *Coup-tfii*, whereas *Rarb*, *Rarg*, and *Rxr* are highly-restricted [346], which resembles the pattern of expression of *Hnf4a* (see Chapter 2). The co-existence of RARα/RXRα, HNF4α, and COUP-TFII in the liver and their capacity to bind to *Gck* promoter confer the change of expression of *Gck* under different nutritional and hormonal stimuli, as evidenced by the fact that RA can induce a dynamic change in the binding of these nuclear receptors to the *Gck* promoter (Fig. 4.17). The occupancy of the specific NRs at this RARE under specific conditions may be a direct reflection of the nutritional and hormonal states of the liver, an organ constantly integrating the internal and environmental signals for achieving glucose and lipid homeostasis.

CHAPTER V
CONCLUSIONS AND FUTURE DIRECTION

Conclusions

In this dissertation, we have shown that retinoids, metabolites of vitamin A (VA), can synergize with insulin to induce the expression of *Srebp-1c* and *Gck* in primary rat hepatocytes. Retinoid-induced expression of *Srebp-1c*, due to the activation of RXR, results in increased expression of SREBP-1c target gene, fatty acid synthase. We demonstrate that retinoic acid responsive elements (RAREs) in the *Srebp-1c* promoter overlap previously identified two liver X receptor elements that mediate insulin action. Moreover, we have characterized the promoter of hepatic *Gck* and identified a RARE using serial deletion reporter gene assays and linker-scan analyses. This RARE overlaps a putative binding site for HNF4α. We show that nuclear receptors RARα, RXRα, HNF4α, and COUP-TFII are capable of binding to the RARE and their binding activities can be modulated by RA, the physiological active form of VA. In addition, the expression of retinoids- and insulin-responsive genes is profoundly modulated by recombinant adenovirus-mediated over-expression of these nuclear receptors. We conclude that VA can control hepatic glucose and lipid metabolism via regulation of the expression of genes involved.

Future Direction

Since the discovery of RARs by P. Chambon and R. M. Evans and their respective co-workers in 1987, we have witnessed a new era of VA research. Currently, there is general agreement that not only the VA status, but also the metabolism of VA, plays critical roles in regulating metabolic homeostasis by activating specific genes to stimulate the cells to produce specific proteins or to inhibit the expression of other genes.

In this dissertation, we tried to answer the question how retinoids regulate nutrient metabolism using *Srebp-1c* and *Gck* as model genes. The analyses of *Srebp-1c* promoter reveal that insulin and RA converge at the same site of *Srebp-1c* promoter and result in a synergistic induction of mRNA expression of *Srebp-1c*. However, we could not identify an insulin responsive element (IRE) at the hepatic *Gck* promoter, which prevents us from further

understanding the mechanism of the synergistic effect between RA and insulin on the induction of hepatic *Gck* transcription. Is there any interaction between the transcriptional factors binding to the RARE and those binding to the IRE, if such an IRE does exist? A possible solution is to integrate serial deletions of hepatic *Gck* promoter into the genome of a cell line that is responsive to insulin and to characterize the promoter activity upon insulin stimulation. Ideally, deletions of promoter containing the IRE can drive the expression of a specific marker. This will allow a direct comparison of constructs within the same genomic context and a systematic and quantitative assessment of the promoter. Alternatively, transgenic mice bearing these promoters can be produced and compared in terms of their response to fasting and re-feeding cycle. However, a potential problem is that sites of integration of the transgenic constructs in the genome, which may affect the regulation of the gene. Another limitation is that unequal number of copies being integrated into different locations in the genome may cause problems in the interpretation of the data obtained.

Although RARα, RXRα, HNF4α, and COUP-TFII can bind to the RARE and regulate *Gck* transcription, the quantitative contribution of each NR is not known. In addition, how is the expression of hepatic *Gck* controlled by these NRs *in vivo*? Since RA can induce a dynamic change in the binding of these NRs to the promoter, it evokes the question that what the effects of other nutritional and hormonal stimuli are on the promoter context. For example, whether insulin alone or insulin together with RA can induce a dynamic change in the binding of these NRs to the *Gck* promoter in primary hepatocytes is under investigation in our lab. Additionally, is there any change in the binding of these NRs to the promoter during the transition from healthy status to metabolic disorder? Other than nutritional and hormonal stimuli, what else can regulate the dynamic change in the binding of these NRs to the hepatic *Gck* promoter? Does circadian clock play a role? Answers to these questions will help further our understanding of the physiological functions of these NRs in the integrated regulation of hepatic *Gck* transcription.

The human physiology is so complex that disruption of one gene or pathway will result in systemic changes to compensate for unbalanced homeostasis. In addition, unregulated overexpression of transgenic gene products may have unwanted physiological or toxic effects. Therefore, transgenic mice bearing the Tet-Off or Tet-On expression systems may be appropriate tools to study the physiological functions of these NRs in regulating hepatic *Gck* transcription. Expression of the NRs in these mice can be regulated both reversibly and quantitatively by exposing them to varying concentrations of tetracycline, or its derivatives such as doxycycline. Ideally, mice with different expression levels of these NRs will respond differentially to fasting and re-feeding cycles or dietary signals (e.g. VAD and VAS) in terms of hepatic *Gck* expression. Alternatively, the relative contribution of each of these NRs to the *Gck* transcription can be quantitatively assessed by co-transfection experiments, in which a combination of varying amounts of expression plasmids containing each of the NRs, together with hepatic *Gck* promoter, are co-transfected into primary hepatocytes. A comparison of *Gck* promoter activities upon stimulation will reflect the relative contribution of each of these NRs.

The general pathways involved in *Gck* expression are beginning to be understood. However, a lot more needs to be done to decipher the molecular mechanisms of this transcriptional regulation.

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